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Virus Sensitivity to Chlorine Disinfection of Water Supplies

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FOREWORD

The Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimony to the deterioration of our natural environment. The complexity of that environment and the interplay between its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems for the prevention, treatment, and management of wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, for the preservation and treatment of public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research; a most vital communications link between the researcher and the user community.

In this study, the effects of virus type, suspending medium and the interaction of these, on the kinetics of virus inactivation by chlorine was examined. The varying effects observed with different viruses indicate that it would be difficult to select a single virus for use as a disinfection indicator under all circumstances.

Francis T. Mayo, Director
Municipal Environmental Research
Laboratory

ABSTRACT

The efficiency of chlorine disinfection of viruses is likely to be affected both by the type of virus and by the nature of the suspending medium. The purpose of this study was to examine the effects of virus type, suspending medium and the interaction of these, on the kinetics of inactivation by chlorine. Six enteric viruses (picornaviruses) as well as SV40 and Kilham rat virus were studied under carefully controlled laboratory conditions. It was found that the different virus types showed a wide range of sensitivity to chlorine disinfection. The rate of inactivation was greater at pH 6 than at pH 10; however, the relative sensitivities of the different viruses were affected differently by changes in pH. This indicates an effect of pH both on the species of chlorine and on the sensitivity of the virus. The presence of dissolved ions also had an effect on sensitivity to chlorine. The possible effects of virus aggregation and the appearance of chlorine resistant mutants were investigated as well. The results indicate that it will be difficult to obtain a single virus type which will serve as a suitable indicator of disinfection under all circumstances.

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SECTION 1

INTRODUCTION AND OBJECTIVES OF STUDY

The purpose of disinfecting potable water supplies is the destruction of pathogenic organisms and thus the elimination and prevention of waterborne disease. Although disinfection may be accomplished in different ways, the most widely used disinfectant today is chlorine, primarily because of its effectiveness, economy, and ease of handling.

Factors affecting the efficacy of chlorine disinfection include: 1) the nature, distribution, and concentration of chlorine and its reaction products in the water to be disinfected; 2) the nature and condition of the water to be disinfected; 3) the temperature of the water; 4) the time of contact between the pathogenic organism and the chlorine; and 5) the nature of the organisms to be destroyed and their concentration, distribution, etc. in the water.

Chlorine, when applied to a water supply, may react with various constituents in the water to form a variety of chlorine species with different disinfecting efficiencies. For example, chloramines, primarily monochloramine and dichloramine, will be formed if the water contains ammonia. These species of chlorine are referred to in practice as combined available chlorine. In the absence of ammonia, organic nitrogen compounds, reducing agents, etc., chlorine may exist in water as either hypochlorous acid (HOCl), hypochlorite ion (OCl^-), or free chlorine, depending upon the pH of the water. Chlorine existing as HOCl or OCl^- is termed free available chlorine. Of the two species, HOCl demonstrates the higher disinfecting efficiency. Compared with free available chlorine, combined available chlorine is much less efficient in terms of disinfection. An increased efficiency will also be achieved with increasing concentrations of the chlorine species.

Further, the nature of the water with respect to other constituents, such as suspended solids, will affect disinfection efficiency. Another factor is temperature; disinfection is more rapid with increasing temperature of the water. Disinfecting efficiency also increases with increased time of contact or exposure.

The final factor governing disinfection efficiency is the nature of the pathogenic organisms present. Data exist which indicate that the resistance of pathogens to free available chlorine varies from one organism to another. For example, spore forming bacteria, such as *Bacillus anthracis*, are more resistant than the non-spore forming bacteria.[1] Fortunately, the most common enteric pathogens do not include spore forming bacteria. The

bacterial pathogens of intestinal origin which cause typhoid fever, cholera, and bacterial gastroenteritis can be inactivated by disinfection with chlorine. Because their resistance is approximately the same or somewhat less than *Escherichia coli* or the coliform organisms, this latter group is useful as an indicator of effective disinfection. The cysts of *Entamoeba histolytica*, on the other hand, are quite resistant to chlorine. However, they can be removed effectively by sand filtration.

A review of the literature indicates that a number of enteric viruses are resistant to inactivation by chlorine; in fact, some are reported to be significantly more resistant to chlorine than *E. coli*. Since the major purpose of this research was to determine the sensitivity of certain enteric viruses to chlorine, the existing information on the inactivation of virus by chlorine will be reviewed in greater depth.

Early study on the viricidal action of chlorine indicated that the amounts of chlorine required to inactivate virus were considerably greater than are routinely used in the treatment of potable water supplies.[2,3] In what was probably the first well defined study, Clarke and Kabler[4] found coxsackievirus A2 to be quite resistant to free available chlorine. It was concluded that the amount of free available chlorine required to inactivate coxsackievirus A2 was between 7 and 46 times that required to kill *E. coli*. On the other hand, it was found in a subsequent study that adenovirus type 3, *E. coli* and *Salmonella typhosa* were all inactivated or destroyed at approximately the same concentration of free chlorine.[5]

In 1958, Weidenkopf[6] reported that 99 percent inactivation of poliovirus type 1 was obtained with 0.10 mg/ℓ free chlorine in 10 min at a pH of 6.0 and a temperature of 0°C. For the same degree of inactivation, increasing the pH to 7.0 increased the reaction time by approximately 50 percent. Both Weidenkopf[6] and Clarke *et al.*[5] indicated that an increase in pH from 7.0 to 8.8 or 9.0 increased the inactivation period about six times. In comparing these studies,[4,5,6] Clarke *et al.*[7] concluded that at 0-6°C, poliovirus type 1 and coxsackievirus A2 were considerably more resistant to HOCl than *E. coli* while adenovirus type 3 was more sensitive. For 99 percent destruction of *E. coli*, a 99 sec contact time was required at 0.1 mg/ℓ chlorine as HOCl. The same percentage of the adenovirus was inactivated in about one-third the time with the same concentration of HOCl. Under the same conditions, 8.5 min was required to inactivate 99 percent of the poliovirus, i.e., five times the contact time required for *E. coli*. Coxsackievirus required a contact time in excess of 40 min, more than 24 times that required for *E. coli*.

Kelly and Sanderson,[8] in studying six different enteric viruses, found that each virus possessed a different sensitivity to chlorine. The results of their study suggested that the inactivation of enteric viruses in water at pH 7.0 and 25°C required a minimum free residual chlorine concentration of 0.3 mg/ℓ with a contact time of at least 30 min. With combined chlorine in water, it was determined that a concentration of at least 9.0 mg/ℓ was necessary for a 99.7 percent inactivation of poliovirus at 25°C and a pH of 7.0.[9] These studies and others have demonstrated that combined chlorine residuals (chloramines), as well as the hypochlorite ion (OCl^-), are much

less effective in inactivating virus than hypochlorous acid (HOCl).

In the case of the studies performed by Kelly and Sanderson,[8,9] it would appear that the viricidal efficiency of HOCl is more than 50 times greater than that of the chloramines. However, an investigation reported by Scarpino *et al.*[10] showed that the hypochlorite ion (OCl^-) was more effective in inactivating poliovirus type 1 than hypochlorous acid (HOCl). In fact, OCl^- was seven times more effective than HOCl. This observation is contrary to the findings of others,[4,6,9] and to the generally accepted understanding of chlorine disinfection. It was indicated by the authors that the borate-KCl-NaOH buffer used may have caused the unusual HOCl-OCl^- effect.

There have been a number of other studies of a similar nature which have provided additional information on the inactivation of viruses by chlorine. In many instances, these studies have produced inconsistent results. The reason for this is not clear but part of the explanation may be related to the different experimental procedures and conditions that have been used. Since it is not intended that this be an exhaustive literature review, these studies will not be discussed. Further, they are of marginal importance to the following research.

It would seem appropriate, however, to conclude this review by citing the detailed study performed by Liu *et al.*[11] In discussing the question of inactivation of viruses by chlorine, the authors point out that, of the studies performed since 1950, only about nine enteric viruses have been tested as to their response to chlorine while over 100 enteric viruses have been described. Considering all the available data, it was concluded that there was sufficient evidence to indicate that a range of differences in resistance to chlorine existed among the viruses examined. Except for adenovirus type 3, most viruses tested have shown a resistance to chlorine about ten times greater than the enteric bacteria.

With this background Liu and his co-workers examined 20 strains of human enteric viruses as to their response to free chlorine. The experiments were performed by adding individual viruses to Potomac River water which was partially treated by coagulation with alum and filtration through sand. Chlorine was added at one dosage, 0.5 mg/ℓ, to water having a pH of 7.8 and stored at 2°C. Samples for viral assay were withdrawn with time, corresponding to different contact times. To illustrate the wide range of resistance to chlorine of the viruses studied, the authors point out that the most sensitive virus was reovirus type 1 which required 2.7 min for inactivating 4 logs (99.99 percent) of the virus with 0.5 mg/ℓ of free chlorine. The most resistant was poliovirus type 2 which required 40 min for the same degree of inactivation. It would appear that this conclusion is based on extrapolating the experimental data using first order kinetics. On the other hand, using actual experimental data, the most resistant virus would seem to be echovirus type 12 which required a contact time of greater than 60 min for 99.99 percent inactivation.

The authors conclude from their extrapolated values that, "as groups, the reoviruses are definitely the least resistant to chlorine treatment;

both adenoviruses and echoviruses are relatively less resistant; and the polioviruses and coxsackieviruses are the most resistant." However, if a 20 min contact time is arbitrarily assumed, most of the viruses tested would have been 99.99 percent inactivated at a free residual of 0.5 mg/ℓ, according to the authors. Although these results are meaningful, they should not be considered the final answer.

Water supply personnel cannot afford to become complacent regarding the elimination of enteric viruses in potable water supplies. For example, although most of the viruses studied by Liu *et al.*[11] would be inactivated with a free chlorine residual of 0.5 mg/ℓ and a contact period of 20 min, there were still 20 percent of the viruses tested which showed resistance to this condition of chlorination. It may be pointed out that there have been instances where viruses have been reported as being detected in finished water supplies. For example, viruses were detected in samples of water obtained from the distribution system of two out of three New England communities examined.[12]

Because of the significance of the results presented by Liu *et al.*,[11] coupled with the importance associated with the question of viruses in public water supplies, their observations should be independently confirmed. It was the purpose of the following research to do just this and, at the same time, to obtain additional information which will provide further insight and knowledge regarding the inactivation of enteric viruses by chlorine. Since it has been observed that viruses differ greatly in their sensitivity to chlorine disinfection, and because the presence of viruses in water supplies may well prove to be a major public health problem, it is of critical importance to answer the following questions:

1. What is the full range of resistance of viral pathogens to chlorine?

2. How much is the effectiveness of chlorination affected by the quality of the suspending medium, e.g., pH, temperature, ionic strength?

3. Can genetic selection produce more resistant viral strains?

Answers to these questions should provide knowledge of the effectiveness of the current practice of chlorination in dealing with waterborne viruses and a rational basis for improving chlorine disinfection.

SECTION 2

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

1. The viruses studied demonstrated a wide range of sensitivity to free available chlorine. This was true in comparing viruses which are closely related, i.e., picornaviruses, and even for different types of the same virus, i.e., poliovirus types 1 and 2.

2. For the six (6) picornaviruses examined, the rate of inactivation by free available chlorine at pH 10.0 was less than at pH 6.0, providing the other variables remained the same.

3. The rank ordering of the viruses with respect to their relative sensitivity to free available chlorine was different at pH 6.0 and at pH 10.0, indicating that changes in pH have differing effects on different virus types as well as on the chlorine species.

4. Potassium, calcium and magnesium ions can significantly affect the rate of chlorine inactivation of poliovirus type 1.

5. At 2°C, the inactivation rate of poliovirus type 1 was observed not to vary linearly with different free available chlorine concentrations.

6. Kilham Rat virus and SV40 were found to be more sensitive to inactivation by free available chlorine than the picornaviruses.

RECOMMENDATIONS

1. Since different viruses vary in their sensitivity to chlorine, caution should be exercised in using a given virus to determine the efficacy of disinfection, i.e., chlorination. This observation may be valid for other disinfectants, so unless the most resistant virus is known for a given condition, the true efficacy of a disinfection process in terms of virus inactivation cannot be determined reliably.

2. The effect of various inorganic ions, alone and in combination, on the rate of virus inactivation by various disinfectants should be thoroughly investigated as such information might relate to the disinfection of natural waters.

3. The occurrence of disinfectant resistant viruses, resulting from genetic selection caused by the disinfectant itself, should receive added attention.

SECTION 3

MATERIALS AND METHODS

PREPARATION AND PURIFICATION OF STOCK VIRUSES

Six of the eight viruses used in the chlorine disinfection experiments were enteric picornaviruses. Five of these viruses, poliovirus type 2 (Lansing), coxsackievirus type A9 (Griggs), coxsackievirus type B5 (Faulkner), echovirus type 1 (Farouk), and echovirus type 5 (Noyce), were obtained from the National Institute of Health, Bethesda, MD. The sixth picornavirus, poliovirus type 1 (Mahoney), was obtained from Dr. Gerald Berg, Environmental Protection Agency, Cincinnati, OH. All six of these viruses were cultured on and assayed by the plaque technique using Buffalo Green Monkey (BGM) kidney monolayers.

The BGM cells were grown in medium 199 (Grand Island Biological Co., Grand Island, NY) containing 10 percent fetal calf serum (FCS) (Grand Island Biological Co., Grand Island, NY) and maintained in medium 199 containing 5 percent FCS after reaching confluence on the fifth day. To each 100 mℓ of medium, 1.0 mℓ of an antibiotic solution was added. This antibiotic solution contained penicillin, 10,000 μ/mℓ; fungizone, 25 mcg/mℓ; and streptomycin, 10,000 mcg/mℓ; all of these antibiotics were obtained from Grand Island Biological Co., Grand Island, NY.

Each virus was purified by inoculating a dilute virus suspension onto a BGM monolayer, growing it up, and isolating a single plaque. The isolated plaque was serially passaged in BGM cells until stocks with adequate titers were obtained (10^{-6} to 10^{-8} PFU/mℓ). The plaque-purified stocks were subjected to a freeze-thaw procedure three times, and then treated with Freon 113 and sucrose gradient centrifugation to remove any chlorine demand. Virus stocks were homogenized with Freeon 113 (Trichlorotrifluorethane, E. I. Dupont de Nemours and Co., Wilmington, DE) (3 parts virus to 2 parts Freon 113) in a Sorvall Omnimixer (Ivan-Sorvall Inc., Newton, CT) at a setting of 5 for 2 min.

The homogenized mixture was centrifuged at 2000 rpm in a Sorval centrifuge (Ivan-Sorvall Inc., Newton, CT) for 20 min at room temperature. The supernatant containing virus was removed and an aliquot of 5-6 mℓ was overlayed onto a linear 5-20 percent sucrose gradient. The gradients were centrifuged in a Beckman SW 25.2 rotor at 22,000 rpm for 2 hr (Beckman Instruments, Inc., Palo Alto, CA). Three mℓ fractions were collected from the bottom of the cellulose nitrate-sucrose gradient tube and the highest titered fractions were pooled to obtain a uniform virus stock. Three mℓ

aliquots of the pooled virus were frozen and stored at -70°C until used.

Simian vacuolating virus 40 (SV40), a papovavirus, was obtained from Dr. Lowell Hager, Department of Biochemistry, University of Illinois. SV40 virus was grown and assayed on CV-1 cells, a derivation of African Green Monkey Kidney. The cells were grown in medium 199 containing 2 percent fetal calf serum. The cells reached confluency after one week, at which time they were split into flasks or plates. SV40 was also purified by the Freon 113 method followed by sucrose gradient centrifugation as described above.

Kilham Rat virus, a parvovirus, was obtained along with the Rat Nephroma cells in which they were grown, from Dr. Lois A. Salzman, National Institute of Health, Bethesda, MD. Rat Nephroma cells were grown in medium 199 containing 10 percent fetal calf serum and reached confluence in 7 days at which time they were split and placed into new flasks or plates. Kilham Rat virus was also purified using the Freon 113 and sucrose gradient methods.

VIRUS ASSAY

The titers of the six enteric viruses (polio 1, polio 2, coxsackie A9, coxsackie B5, echo 1, and echo 5) were determined by plaque assay, using confluent BGM monolayers in 60 x 15 mm tissue culture plates (Falcon Division, Becton, Dickinson and Co., Oxnard, CA). The virus was diluted in Hank's balanced salt solution (Grand Island Biological Co., Grand Island, NY) containing 2 percent fetal calf serum, and 0.5 mℓ of each dilution was inoculated in duplicate onto BGM plates. After incubation at 37°C under 5 percent CO_2 for 1-2 hr, a 5-6 mℓ aliquot of overlay medium consisting of medium 199 plus 10 percent fetal calf serum, antibiotics and 0.9 percent agar was placed on top of the BGM monolayer and incubated at 37°C until the appearance of plaques. At this time the monolayer was fixed with 70 percent ethanol, formaldehyde and acetic acid in a volume ratio of 20:2:1. The monolayer was then stained with a 1 percent solution of crystal violet and the plaques were counted.

Determination of the titer of SV40 was also done by plaque assay, except that the assays were performed on CV-1 monolayers. The virus was diluted in phosphate buffered saline (PBS) and 0.2 mℓ of each dilution was plated in duplicate. After a 2 hr incubation at 37°C, a 5 ml overlay containing 0.9 percent agar, 5 percent fetal calf serum, 0.15 percent $NaHCO_3$ and antibiotics in medium was added. After 5 days of incubation at 37°C, another 5 mℓ of the above overlay was added to each plate. Plaques did not appear until the 13th or 14th day, at which time a 3 mℓ staining layer consisting of 0.9 percent agar, 0.005 percent neutral red, 0.15 percent $NaHCO_3$ and antibiotics in medium 199 was added. The final count of plaques was performed after 16 days.

Use of the plaque assay technique was not possible for the Kilham Rat virus (KRV) for technical reasons and therefore this virus was assayed by the hemagglutination technique (HA). Kilham Rat virus will only proliferate in cells which are actively growing. Therefore, the KRV was grown in cells which had only recently been sparsely plated, and were therefore actively

dividing. The KRV was diluted in PBS and 0.1 mℓ of each dilution was added in quintuplicate onto sparsely plated but attached Rat Nephroma cells (5×10^5 cells/60 mm culture dish) and allowed to incubate for 2 hr at 37°C under 5 percent CO_2. During the 2 hr incubation period, the culture dishes were gently rocked every 15 min. After the 2 hr incubation period, 5 mℓ of medium 199 with 10 percent fetal calf serum was added to each plate and they were incubated for 6 days. By the 6th day, cytopathic effect was apparent at lower dilutions.

The cells were scraped off the culture dishes with a rubber policeman; the collected cells were then subjected to a freeze-thaw procedure three times. An aliquot of 25 µℓ of the lysate was placed in the first well of a microtiter plate in which there was 25 µℓ of PBS. Serial two-fold dilutions were then made and 25 µℓ of a 1 percent solution of guinea pig red blood cells (diluted in PBS) was added to each well. The microtiter plates were incubated at room temperature for 1 hr and then the HA titers were recorded as the highest dilution giving agglutination.

PREPARATION OF CHLORINE WATER AND CHLORINE DEMAND FREE BUFFER

Stock solutions of chlorine were prepared using Clorox (Clorox Corp., Oakland, CA), as these solutions proved to be more stable than those prepared from chlorine gas. To compare the two chlorine solutions (Clorox vs. chlorine gas), an inactivation experiment was performed with polio 1 using both solutions; no difference was observed in the inactivation rate with the two chlorine solutions.

Chlorinated water was prepared by adding 5 mg/ℓ of a hypochlorite solution, prepared from Clorox, to deionized-distilled water and allowing the chlorinated water to stand several days. This chlorinated water was then used to prepare chlorine demand-free buffer (CDF buffer). For experiments performed at pH 6.0 and 7.8, a 0.05 M phosphate buffer was prepared from NaH_2PO_4 and Na_2HPO_4 and the above mentioned chlorinated water. For those experiments performed at pH 10.0, a 0.05 M borate buffer (H_3BO_3-NaOH) without KCl was used; again the buffer was prepared using chlorinated water. Both the phosphate and borate buffers were boiled for several minutes and exposed to ultraviolet light for 48-72 hr to achieve dechlorination. All buffer solutions were analyzed for the absence of chlorine by the orthotolidine test before use.[13] When the orthotolidine test was negative, the buffers were considered to be chlorine demand-free.

EXPERIMENTAL EQUIPMENT AND PROCEDURES

For each experiment, 5 ℓ of the appropriate CDF buffer solution was prepared and sufficient chlorine added to 4 ℓ to obtain the desired residual. The remaining 1 ℓ was set aside to be used in the virus control beaker. A 400 mℓ aliquot of the CDF buffer solution, having the desired chlorine residual, was placed into each of three stainless steel beakers labeled "Test," "Chlorine Control," and "Temperature Control," respectively, and allowed to stand at room temperature for 1 hr. Another beaker labeled "Virus Control" was filled with 400 mℓ of the CDF buffer solution without any added chlorine. This "soaking" of the beakers eliminated any chlorine

demand which might have been associated with the stainless steel beakers. At the end of 1 hr, the contents of each beaker were discarded and replaced with 400 mℓ of the appropriate CDF buffer solution. The beakers were then placed into a circulating water bath and allowed to equilibrate in the constant temperature water bath for approximately 1 hr or until the desired temperature was achieved (Masterline Water Bath and Circulating Pump, Forma Scientific, Marietta, OH).

Before initiation of an inactivation experiment, 200 mℓ was removed from the "Chlorine Control" beaker and titrated with an amperometric titrator (Wallace and Tiernan Titrator, Penwalt Corp., Belleville, NJ) to determine the initial free available residual chlorine level. After determining the chlorine residual, a stirring rod attached to a six-place multiple laboratory stirrer (Phipps and Bird Laboratory Stirrer, Phipps and Bird, Richmond, VA) was placed into each beaker and mixed at maximum speed (approximately 180 rpm); the temperature was allowed to re-equilibrate for approximately 5 min.

Initiation of each inactivation experiment was accomplished by the addition of 1 mℓ of a virus preparation to the "Virus Control" and "Test" beakers. Time-point samples from the "Virus Control" and "Test" beakers were withdrawn in 5 mℓ aliquots at pre-determined time intervals and added to sodium thiosulfate (12 mg/mℓ) and thoroughly mixed to neutralize the chlorine. At the end of each experiment, 200 mℓ of the CDF buffer solution was removed from the "Chlorine Control" and "Test" beakers to determine the chlorine residual level. Dilutions were made of the time-point samples and plated onto BGM cells and allowed to incubate until plaques appeared. The plaques were counted and the results plotted, i.e., percentage virus survival vs. time of exposure to chlorine.

SECTION 4

RESULTS AND DISCUSSION

EFFECT OF pH ON CHLORINE INACTIVATION OF THE SIX PICORNAVIRUSES

The hypochlorous acid (HOCl) produced when chlorine is added to water can be associated (HOCl) or disassociated (OCl^-); the distribution of these two chlorine species is a function of pH. At pH 6.0 and 0°C, about 98.2 percent of the chlorine is in the associated form (HOCl). At pH 10.0 and 0°C, only 0.5 percent of the chlorine is as hypochlorous acid, the remainder is in the form of hypochlorite ion (OCl^-).[14] Of the two chlorine species, HOCl demonstrates the greatest disinfecting efficiency.

Experiments were performed with polio 1 and 2, echo 1 and 5, and coxsackie A9 and B5 at pH 6.0 and 10.0 to evaluate the effect of these two major species of chlorine, which constitute free available chlorine. The six viruses were chosen for their wide range of sensitivities to chlorine, as reported by Liu *et al.*;[11] each of the three pairs of viruses represents a chlorine-sensitive and a chlorine-resistant strain. Chlorine inactivation studies were also performed at pH 7.8 with echo 1, coxsackie B5, echo 5, and polio 1 in order to have data comparable to that of Liu and co-workers. Table 1 summarizes the results, giving the time required for 99 percent (2 logs) inactivation of the six picornaviruses at pH 6.0 and 7.8 in phosphate buffer, and at pH 10.0 in borate buffer. These values were obtained by combining the data from at least three separate inactivation experiments for each virus, and graphically determining the point of 99 percent inactivation. Experimental error was generally less than 20 percent.

All of the chlorine inactivation experiments were performed at 5°C ± 0.2°C. The pH never varied more than five-tenths of a pH unit during a given experiment. The range of pH of the separate experiments is shown in Table 1. The initial virus titer in both the "Virus Control" and "Test" reactors in each experiment was about 1×10^4 PFU/mℓ. The dosage of chlorine employed in each experiment was 0.50-0.53 mg/ℓ. The residual chlorine level, as measured amperometrically in the "Test" reactor after each experiment, often decreased but generally not more than 0.03 mg/ℓ. This decrease represents approximately a 6 percent chlorine-demand by the virus sample. Table 1 shows the range of residual chlorine at the end of each of three separate experiments for each of the viruses.

The results given in Table 1 indicate that there is a significant difference in the time of inactivation for the various viruses at pH 6.0 and 10.0. In every case, the rate of inactivation at pH 10.0 was dramatically

less than at pH 6.0. For example, echo 1 was 99 percent inactivated in 0.5 min at pH 6.0 and in 96 min at pH 10.0, while polio 1 was 99 percent inactivated in 2.1 min at pH 6.0 and 21 min at pH 10.0.

TABLE 1. TIME REQUIRED FOR 99 PERCENT INACTIVATION BY FREE RESIDUAL CHLORINE AT 5.0°C ± 0.2°C

pH	Concentration of* free chlorine mg/ℓ	Virus strain	Minutes for 99% inactivation	Rank ordering
6.00	0.46-0.49	Coxsackie A9 (Griggs)	0.3	1
6.00	0.48-0.49	Echo 1 (Farouk)	0.5	2
6.00-6.02	0.48-0.51	Polio 2 (Lansing)	1.2	3
6.00-6.03	0.38-0.49	Echo 5 (Noyce)	1.3	4
6.00	0.47-0.49	Polio 1 (Mahoney)	2.1	5
6.00-6.06	0.51-0.52	Coxsackie B5 (Faulkner)	3.4	6
		Coxsackie A9 (Griggs)	ND	
7.81-7.82	0.47-0.49	Echo 1 (Farouk)	1.2	1
		Polio 2 (Lansing)	ND	
7.79-7.83	0.48-0.52	Echo 5 (Noyce)	1.8	3
7.80-7.84	0.46-0.51	Polio 1 (Mahoney)	1.3	2
7.81-7.82	0.48-0.50	Coxsackie B5 (Faulkner)	4.5	4
10.00-10.01	0.48-0.50	Coxsackie A9 (Griggs)	1.5	1
10.00-10.40	0.49-0.51	Echo 1 (Farouk)	96.0	6
9.89-10.03	0.48-0.50	Polio 2 (Lansing)	64.0	4
9.97-10.02	0.49-0.51	Echo 5 (Noyce)	27.0	3
9.99-10.40	0.50-0.52	Polio 1 (Mahoney)	21.0	2
9.93-10.05	0.50-0.51	Coxsackie B5 (Faulkner)	66.0	5

*Range of measured free chlorine residual in the "Test" reactor at the termination of each of three separate experiments.

ND = not determined

The rank ordering in Table 1 demonstrates the wide range of sensitivities of related viruses to chlorine disinfection. For example, at pH 10.0, coxsackie B5 was 40 times more resistant than coxsackie A9. Interestingly, there are several cases in which the relative sensitivity to chlorine was altered (rank ordering) between pH 6.0 and 10.0, suggesting important effects of pH on the virion as well as on the chlorine species. Echo 1 was the second most sensitive virus at pH 6.0 but was the most resistant at pH 10.0. Polio 1 was one of the most resistant viruses at pH 6.0; however, it was relatively sensitive at pH 10.0 when compared to the sensitivity of the

other viruses at this pH value. This can be seen most clearly by inspection of Table 2 in which the time required for 99 percent inactivation of the viruses and the ratios of inactivation rates at pH 6.0 and pH 10.0 are compared. Even at pH 7.8, differences in relative sensitivity appeared when rank ordered and compared to results at pH 6.0 (Table 1). Polio 1 and echo 1 were inactivated at approximately the same rate at pH 7.8, while their rates were quite different at pH 6.0. Of particular interest was the observation that polio 1 was inactivated more rapidly at pH 7.8 than at pH 6.0. This suggests that the effect of pH in this range (pH 6.0-7.8) may be greater on the structure and reactivity of the virus than on the species of chlorine.

TABLE 2. COMPARISON OF VIRUS INACTIVATION BY FREE RESIDUAL CHLORINE AT pH 6.0 AND 10.0, AND 5.0°C ± 0.2°C

Virus strain	Minutes for 99% Inactivation		
	pH 6.0	pH 10.0	Ratio*
Coxsackie A9 (Griggs)	0.3	1.5	5
Echo 1 (Farouk)	0.5	96.0	192
Polio 2 (Lansing)	1.2	64.0	53
Echo 5 (Noyce)	1.3	27.0	21
Polio 1 (Mahoney)	2.1	21.0	10
Coxsackie B5 (Faulkner)	3.4	66.0	19

*Time required at pH 10.0 / Time required at pH 6.0

Figures 1 through 6 show the differences in the kinetics of inactivation of the two types of polio, coxsackie and echo viruses at pH 6.0 and 10.0, with 0.5 mg/ℓ of free available chlorine and a temperature of 5°C. Each pair of viruses from the same subgroup is shown on one figure for purposes of comparison and each curve represents the average of three or more separate experiments. It is significant to note that some virus types closely resemble each other in their kinetic response to chlorine while others do not. For example, in Figure 3, coxsackie A9 at pH 6.0 shows a rapid decrease in titer followed by a somewhat slower rate of inactivation, whereas coxsackie B5 appears to be dramatically more resistant to chlorine. The half-life of coxsackie A9 is about 2 sec, whereas the half-life of coxsackie B5 is approximately 0.5 min, a 15-fold difference.

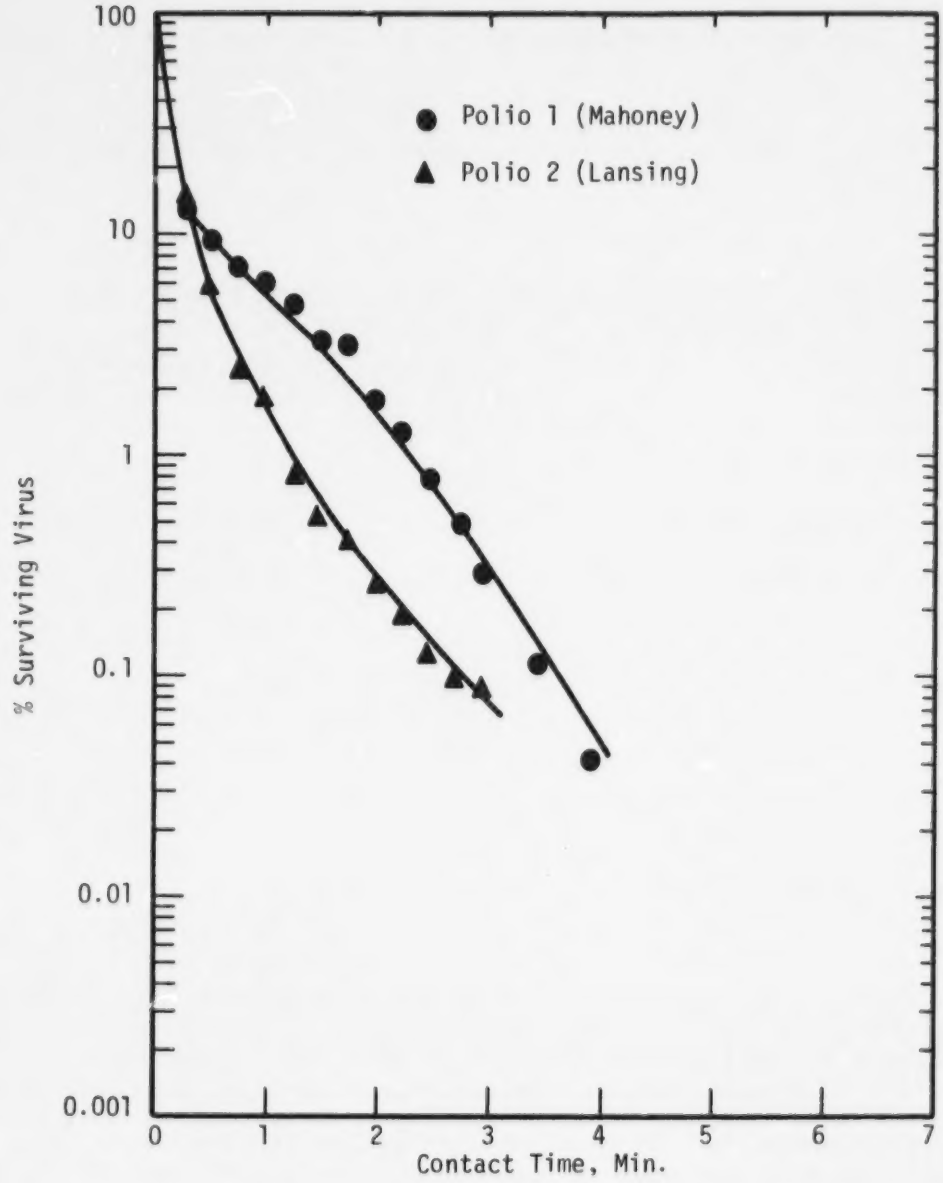


Figure 1. Inactivation kinetics of polio 1 and polio 2 to 0.47-0.51 mg/ℓ free available chlorine at pH 6.0 and 5.0°C.

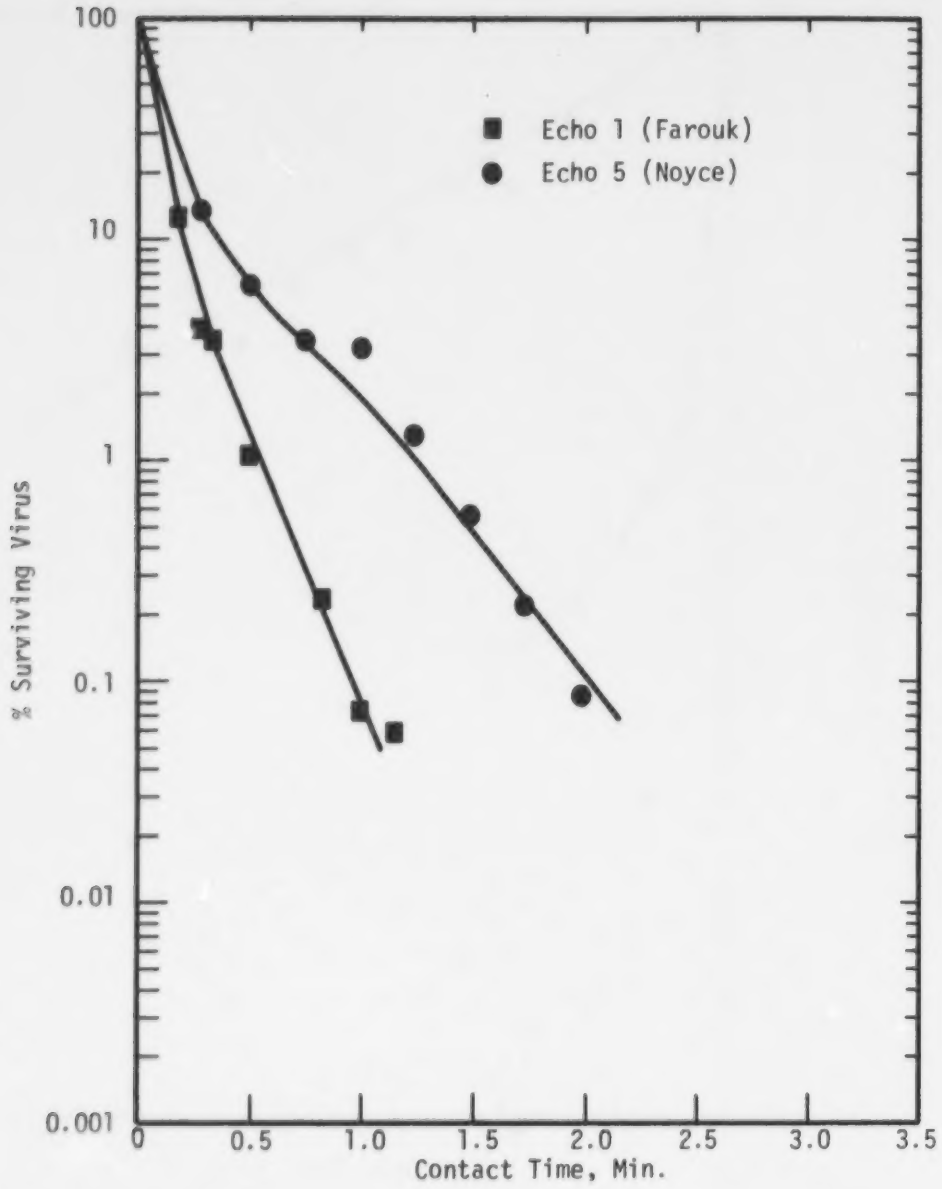


Figure 2. Inactivation kinetics of echo 1 and echo 5 to 0.38-0.49 mg/ℓ free available chlorine at pH 6.0 and 5.0°C.

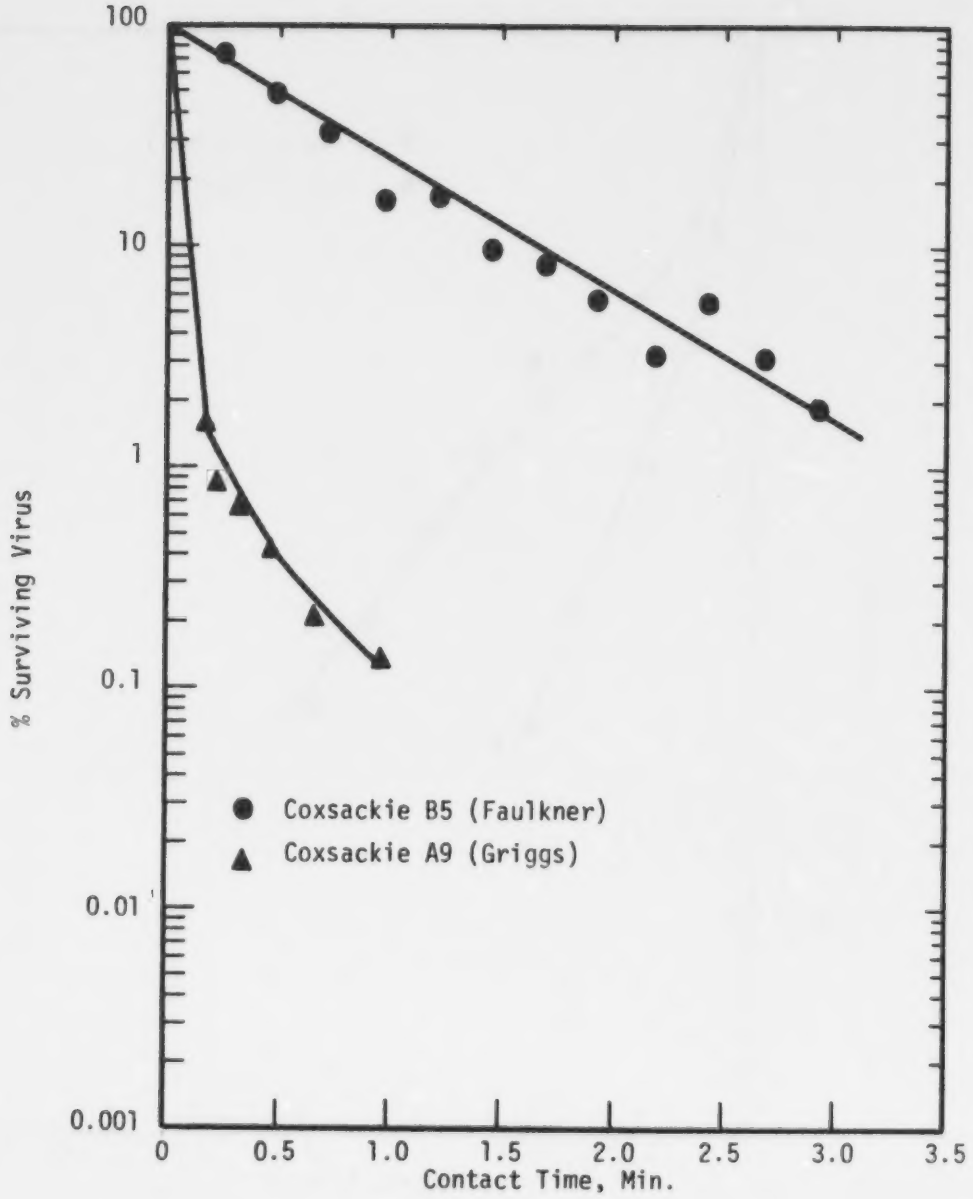


Figure 3. Inactivation kinetics of coxsackie B5 and coxsackie A9 to 0.46-0.52 mg/ℓ free available chlorine at pH 6.0 and 5.0°C.

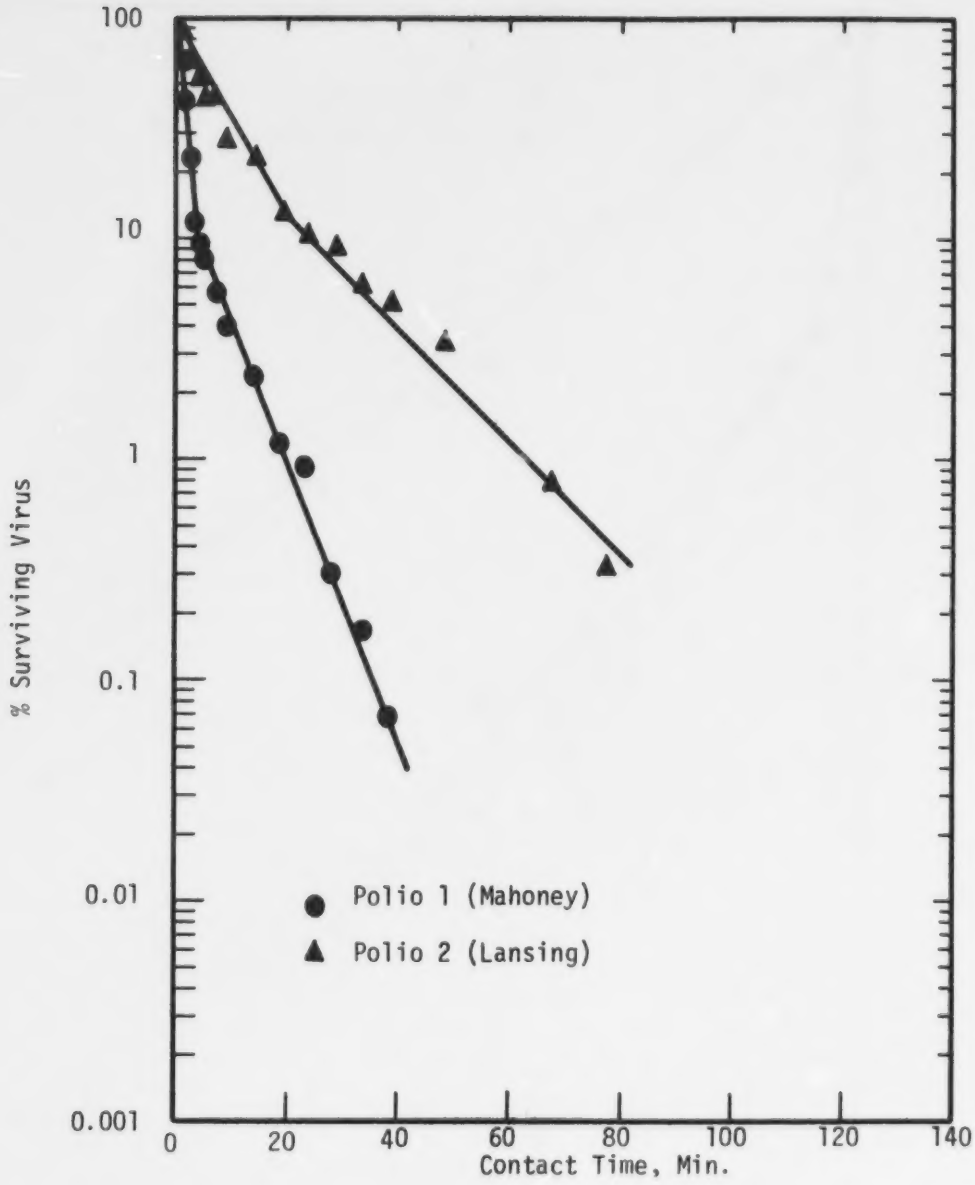


Figure 4. Inactivation kinetics of polio 1 and polio 2 to 0.48-0.52 mg/ℓ free available chlorine at pH 10.0 and 5.0°C.

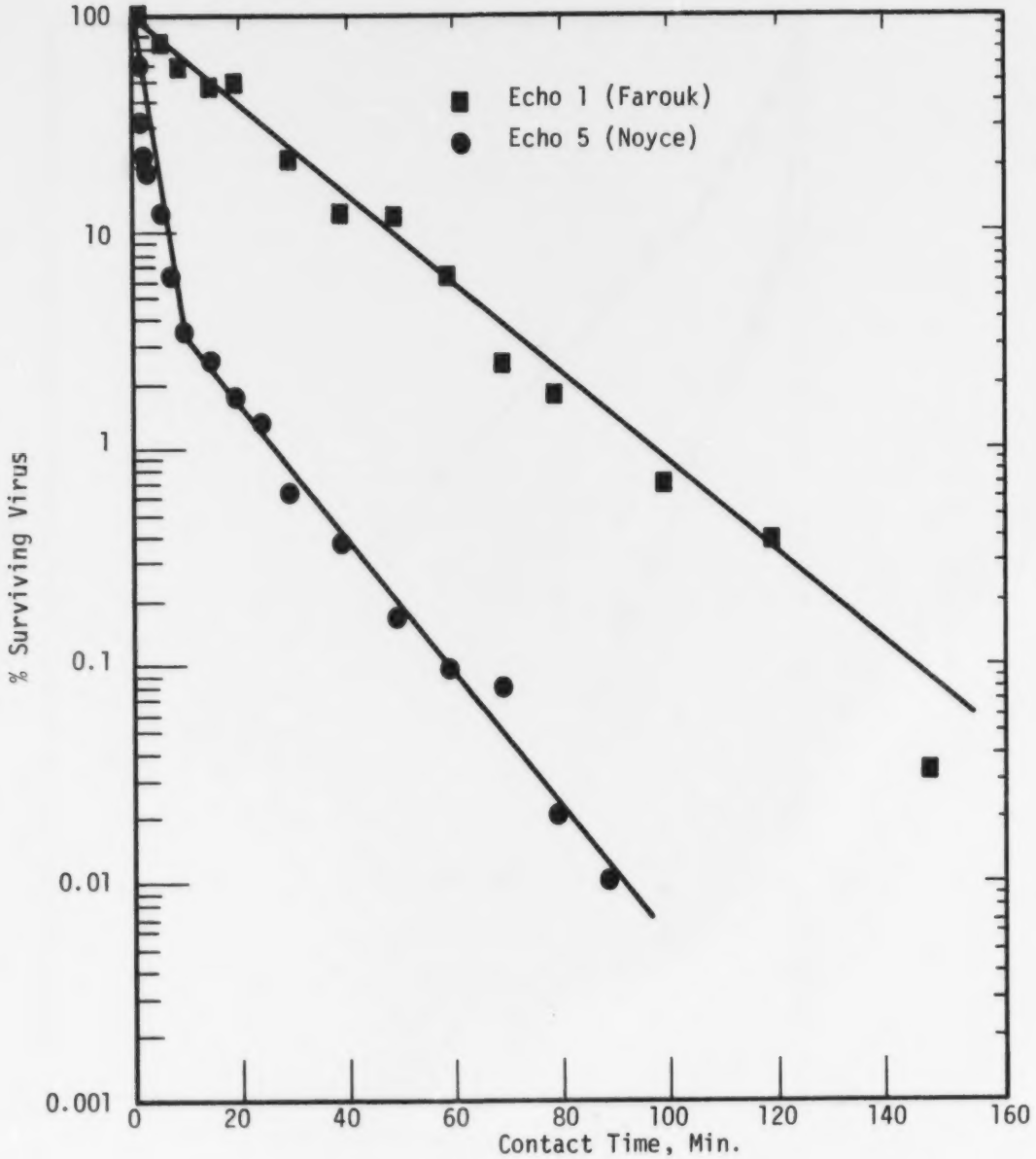


Figure 5. Inactivation kinetics of echo 1 and echo 5 to 0.49-0.51 mg/ℓ free available chlorine at pH 10.0 and 5.0°C.

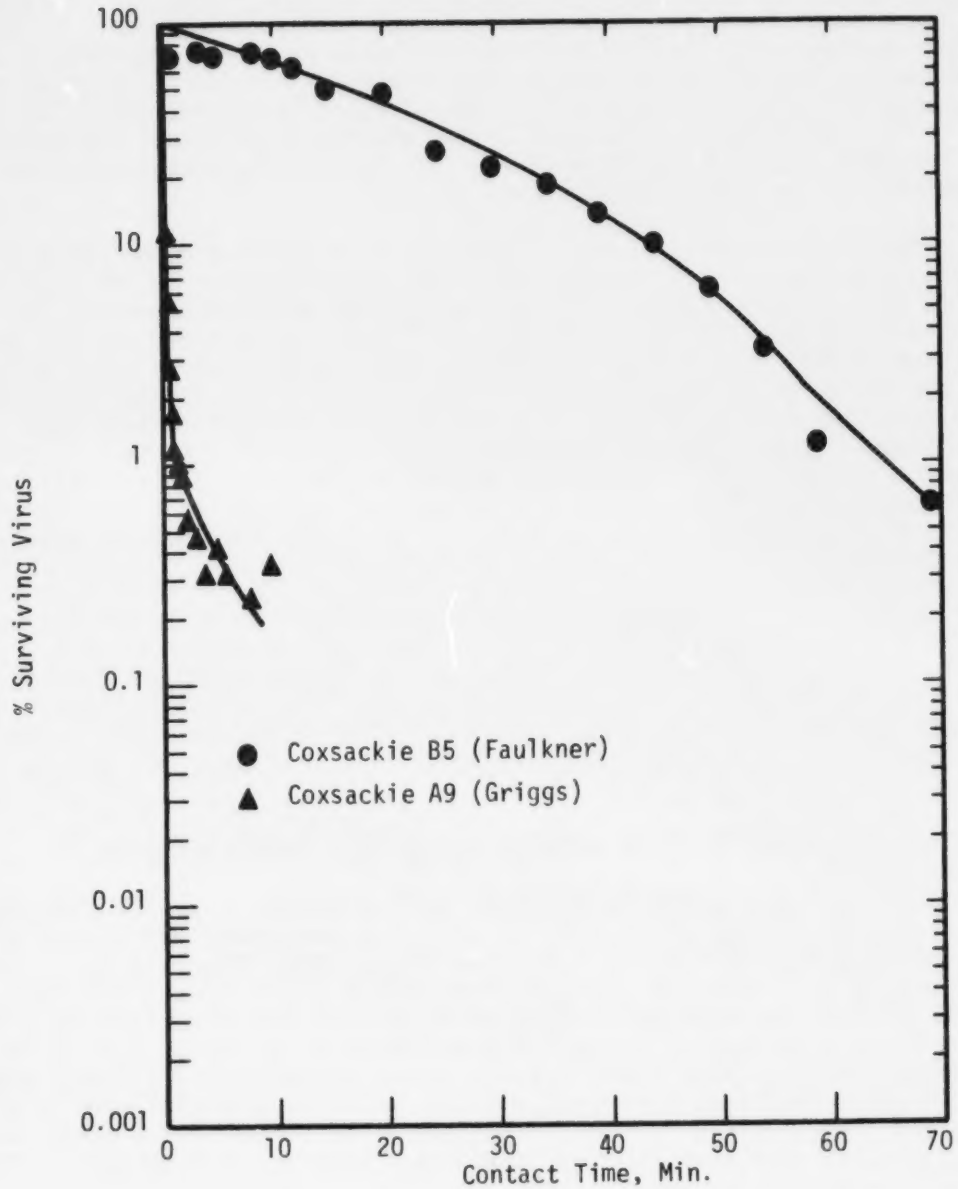


Figure 6. Inactivation kinetics of coxsackie B5 and coxsackie A9 to 0.48-0.51 mg/ℓ free available chlorine at pH 10.0 and 5.0°C.

Figure 1 shows the kinetics of inactivation of polio 1 and 2, at pH 6.0 and 5°C with 0.5 mg/ℓ free available chlorine residual. These viruses display inactivation kinetics which are similar to each other; this is contrary to the results for the two coxsackie viruses (Figure 3). Initially, both polio viruses have a half-life of approximately 8-10 sec. However, a significant difference in the rate of inactivation for polio 1 and polio 2 appeared following approximately 30 sec of exposure to chlorine. Two logs of polio 1 were inactivated after 2 min, whereas polio 2 was inactivated to the same extent in half the time. This is a consequence of the non-linearity of the inactivation curves.

This non-linearity is most likely due to a difference in degree of aggregation between the two viruses, although differences in the rate at which chlorine and the neutralizing thiosulfate solution penetrate the capsid could also contribute to this non-linearity. Limited results using sucrose gradients suggest that virus aggregation amounted to 0.5-8 percent of the particles in the virus stock preparations. This is consistent with the deviation from single-hit kinetics of polio, between 1 and 2 logs inactivation (see Figure 1). Whether strain-specific variations in the degree of aggregation consistently generate differences in inactivation kinetics remains to be determined. The results with polio 1 quantitatively agree with those reported by Weidenkopf,[6] including the biphasic nature of the curve.

Since phosphate buffer was used in preparing the CDF buffer solution at pH 6.0 and borate at pH 10.0, it was important to determine whether the buffers themselves affected the rates of inactivation. Figure 7 compares the kinetics of chlorine inactivation of polio 1 at pH 7.8, using both the borate and phosphate CDF buffer solutions. The curves are indistinguishable indicating that the buffer ions are unlikely to be responsible for the previous results.

CHLORINE INACTIVATION USING CHLORINE DEMAND FREE BUFFER SOLUTION

Chlorine inactivation experiments were performed at pH 7.8 with echo 1, coxsackie B5, and echo 5 in order to have data comparable with Liu *et al.*[11] Figure 8 compares the chlorine inactivation of these three viruses at pH 7.8 with 0.5 mg/ℓ of free available chlorine and at 5°C ± 0.2°C. The times for 99.99 percent inactivation of these three viruses were less than the times reported by Liu *et al.*[11] in their Potomac River water study. Liu *et al.*[11] found, assuming a first order reaction and by extrapolation of their experimental data, that echo 5 required 8.0 min; polio 1, 16.2 min; echo 1, 27.0 min; and coxsackie B5, 39.5 min to achieve 99.99 percent inactivation with 0.5 mg/ℓ free available chlorine in Potomac River water at pH 7.8 and 2°C.

Extrapolation of the data generated in this study indicated that echo 5 required 4.5 min; polio 1, 3.0 min; echo 1, 4.7 min; and coxsackie B5, 6.7 min for the same degree of inactivation of the virus with 0.5 mg/ℓ free available chlorine at pH 7.8 but with CDF buffer solution and at 5°C. Comparing the results of these two independent studies and neglecting the small difference in temperature between the two sets of data, it would

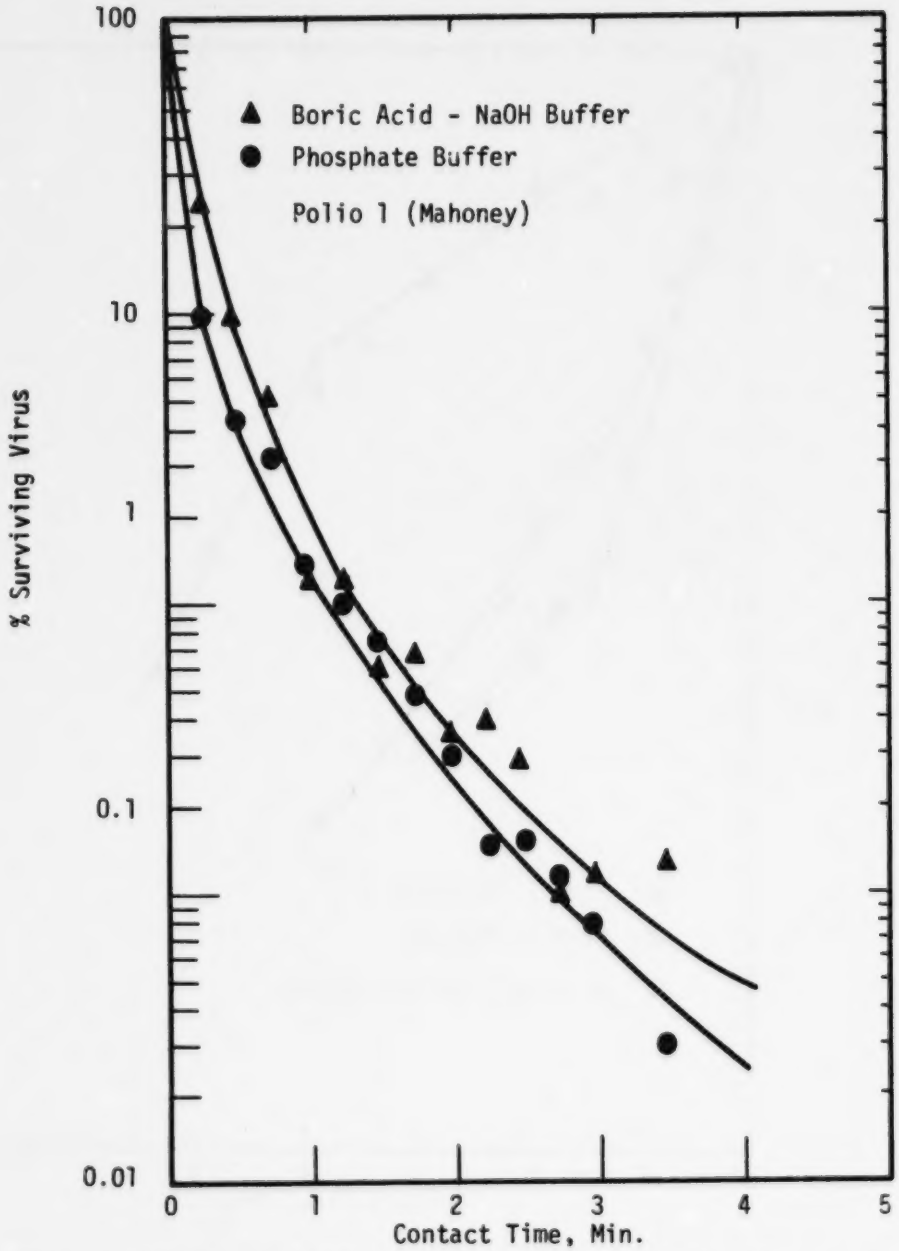


Figure 7. Inactivation kinetics of polio 1 to 0.46-0.51 mg/ℓ free available chlorine in phosphate buffer and boric acid-NaOH buffer at pH 7.8 and 5.0°C.

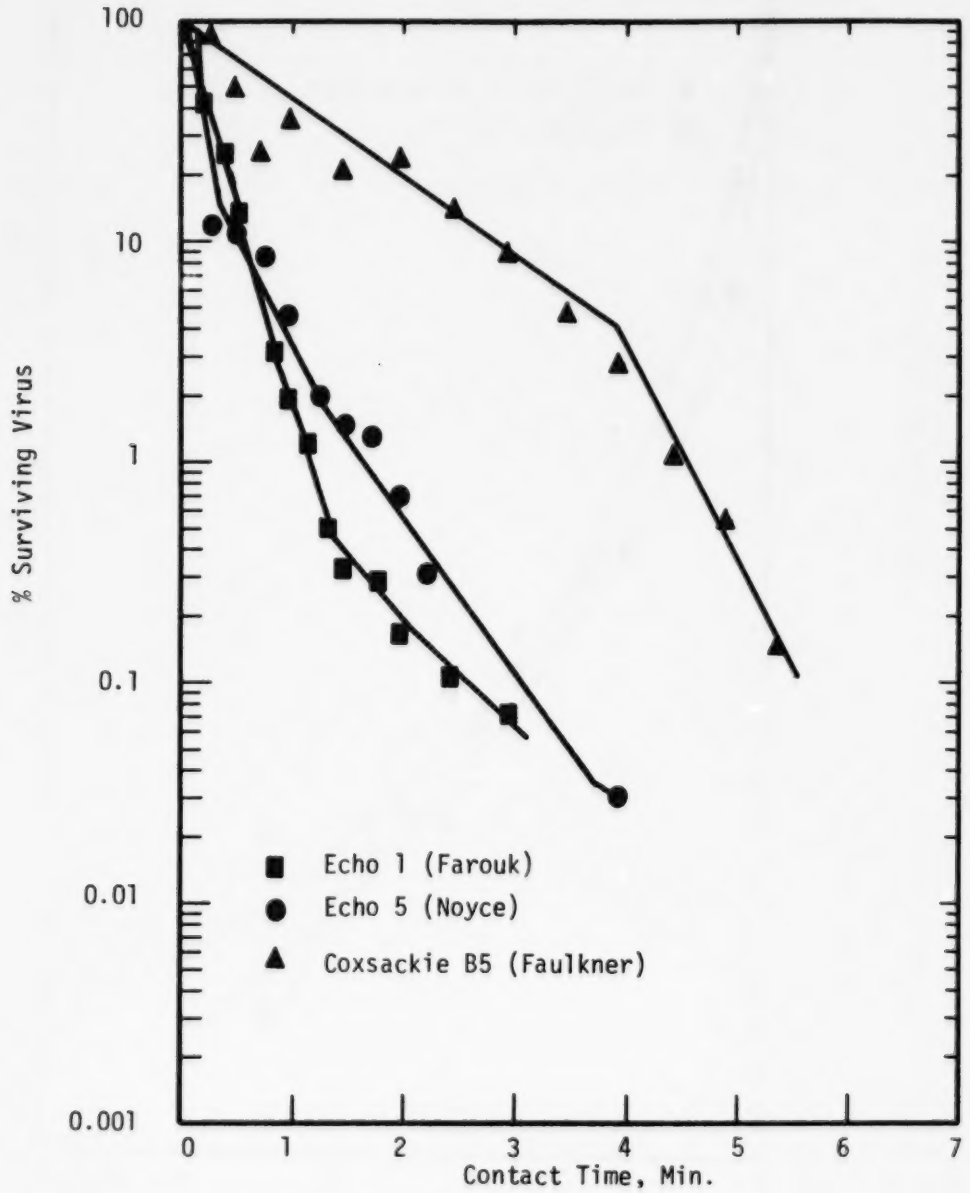


Figure 8. Inactivation kinetics of echo 1, echo 5, and coxsackie B5 to 0.47-0.52 mg/ℓ free available chlorine at pH 7.8 and 5.0°C.

appear that the inactivation of virus in natural water, i.e., Potomac River water, may differ significantly from that which occurs in a chlorine demand free (deionized-distilled water) buffer solution.

CHLORINE INACTIVATION AS A FUNCTION OF VIRUS GROUP

The chlorine sensitivity of different viruses was also considered, particularly with respect to those virus groups which pose a threat to health because of their possible occurrence in water supplies. The picornaviruses, having RNA as their genetic material, appear to demonstrate a wide variation in their sensitivity to chlorine (Table 1). Kilham Rat virus, a parvovirus containing single stranded DNA, was also studied as to its chlorine sensitivity, because it has been speculated that the virus of infectious hepatitis may also belong to this same group of viruses. Table 3 gives the kinetics of inactivation of the Kilham Rat virus at pH 6.0 with 0.5 mg/ℓ free available chlorine and at a temperature of 5°C.

TABLE 3. CHLORINE INACTIVATION OF KILHAM RAT VIRUS AND SV40 VIRUS BY FREE RESIDUAL CHLORINE AT 5.0°C ± 0.2°C

Kilham Rat Virus

Exp. No.	pH	Conc. of free chlorine in test reactor, mg/ℓ		Hemagglutination Titer (H.A. Titer) Control	10 sec	20 sec	30 sec	60 sec
1	6.01	0.48	H.A. Titer	1:1638	1:1792	1:3.2	1:1.6	0
			% Survival	100	109	0.19	0.09	0
2	6.04	0.49	H.A. Titer	1:563	1:243	1:3.2	0	0
			% Survival	100	43	0.56	0	0

SV40 Virus

Exp. No.	pH	Conc. of free chlorine in test reactor, mg/ℓ	% Virus Surviving Control	10 sec	20 sec	30 sec
1	6.01	0.49	100	1.13	0	0
2	6.02	0.48	100	1.67	0.02	0
3	6.02	0.48	100	0.69	0	0

Kilham Rat virus was no more resistant to chlorine than any of the picornaviruses, being 99 percent inactivated in less than 20 seconds. The chlorine sensitivity of SV40 virus, a papovavirus containing double-stranded DNA, was also studied. Table 3 shows the kinetics of inactivation of SV40 virus at pH 6.0 with 0.5 mg/ℓ free available chlorine and at a temperature

of 5°C. The SV40 virus was even more sensitive to chlorine than Kilham Rat virus, being 99 percent inactivated in 10 sec. Even though the SV40 virus has a reputation among tumor virologists as being very hardy under adverse conditions, it appears to be extremely sensitive to chlorine.

EFFECT OF TEMPERATURE AND CHLORINE DOSAGE ON VIRUS INACTIVATION

Chlorine inactivation experiments were performed at 2°C ± 0.5°C and a pH of 6.0 with polio 1 and different levels of free available chlorine residuals, 1μ M (0.07 mg/ℓ), 7μ M (0.5 mg/ℓ) and 28μ M (2.0 mg/ℓ), for comparison with the data reported by Sharp.[15] Sharp observed that at 2°C, pH 6.0, with HOCl, the rate of inactivation of polio 1 increased from about 0.48 log/min with 1μ M of HOCl to twice this rate at 20μ M. However, there was no further increase in the inactivation rate with 40μ M HOCl.[15]

Figure 9 shows the results of inactivating polio 1 with the three chlorine residuals given above; as the level of free available chlorine increases the rate of inactivation increases as well. In Figure 10, the initial rate of inactivation for polio 1 is plotted as a function of residual chlorine. It would appear from Figure 10 that the rate of inactivation does not decrease linearly with the residual chlorine concentration. Below 7μ M of residual chlorine, the rate appears to decrease rapidly in order to intersect the vertical axis at zero residual chlorine concentration. These data are similar to those of Sharp,[15] and are also in agreement with the work of Weidenkopf in his studies at 0°C, as presented by Floyd *et al.*[16] This phenomenon seems to appear only at low temperatures.

EFFECTS OF DISSOLVED IONS ON CHLORINE INACTIVATION

Experiments were performed at 5°C with 0.5 mg/ℓ of free available chlorine, using 0.05 M phosphate buffer (pH 6.0) and boric acid-NaOH buffer (pH 10.0) with and without 0.05 M KCl added, to further investigate the reversal effect of KCl, as observed by Scarpino *et al.*[10] Their results indicated that inactivation of polio 1 was more effective at pH levels where the free available chlorine was predominantly in the form of OCl^- rather than in the form of HOCl. It was reported that inactivation of polio 1 at pH 10.0 was seven times more rapid than at pH 6.0. This is contrary to the findings of other investigators. The buffer used by Scarpino *et al.*[10] in their pH 10.0 experiments was 0.05 M borate buffer (H_3BO_3-KCl-NaOH). The pH 10.0 buffer system used in this study was also boric acid and sodium hydroxide, but with and without added KCl.

As seen in Figure 11, the time required for 99 percent inactivation of polio 1 by 0.5 mg/ℓ of free residual chlorine at pH 10.0 was decreased approximately 50 times by the addition of 0.05 M KCl to the boric acid-NaOH buffer system. The rate of inactivation of polio 1 in 0.05 M phosphate buffer at pH 6.0, with and without 0.05 M KCl, was also studied (Figure 12). Once again, the addition of KCl decreased the time for 99 percent inactivation of polio 1; the decrease was six-fold.

It appears that the presence of KCl in either the boric acid-NaOH buffer, or in the phosphate buffer system, increases the rate of

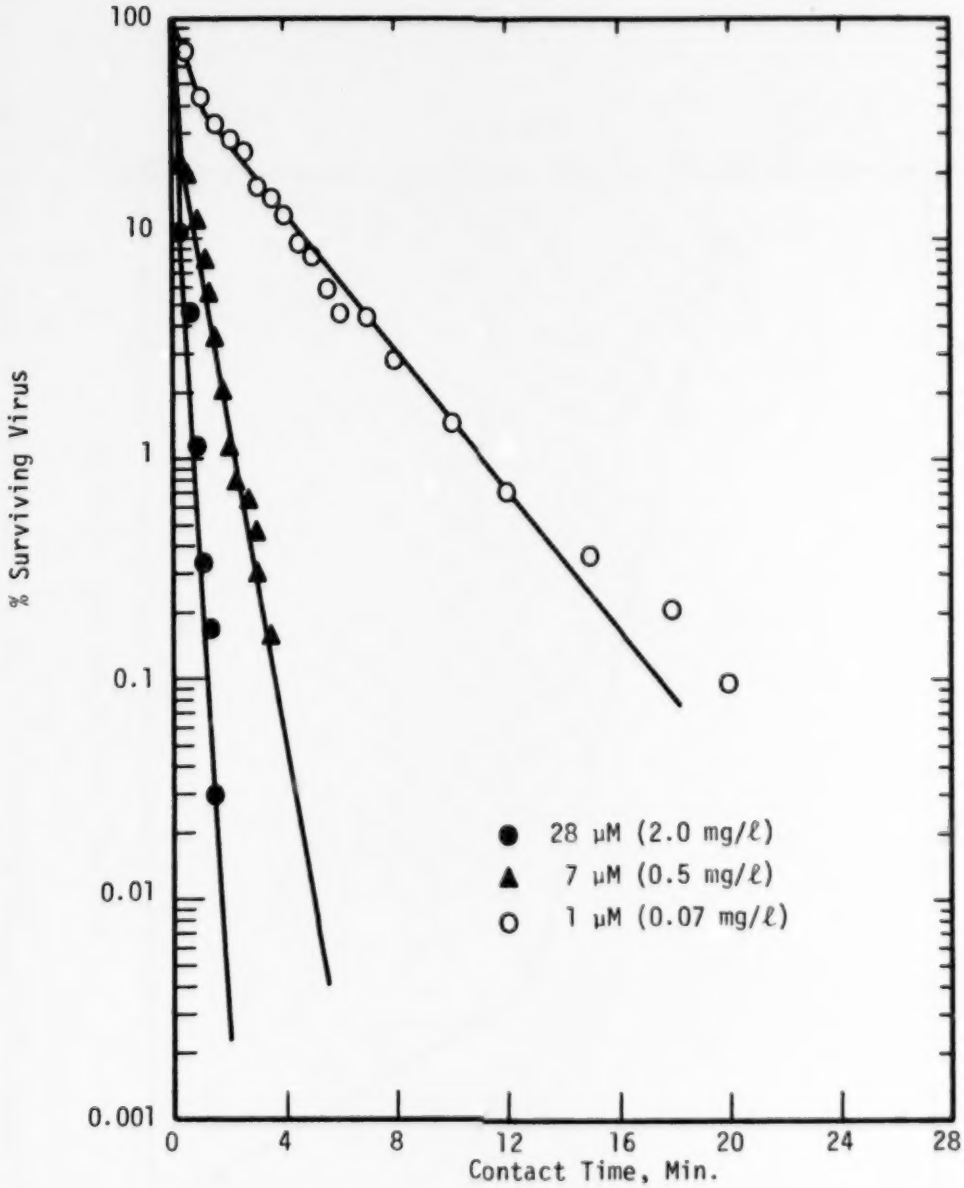


Figure 9. Inactivation kinetics of polio 1 with three levels of free available chlorine at pH 6.0 and 2.0°C.

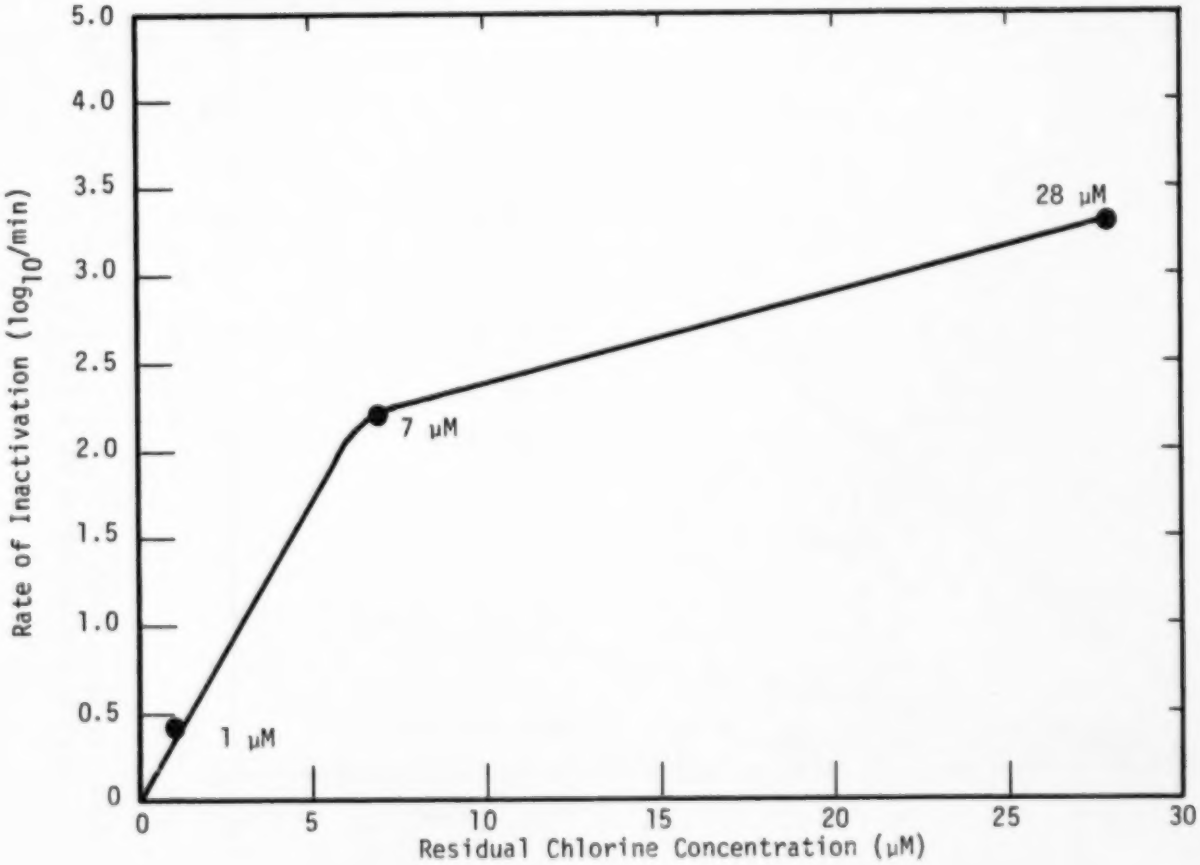


Figure 10. Residual chlorine concentration vs. rate of inactivation of polio 1 at pH 6.0 and 2.0°C.

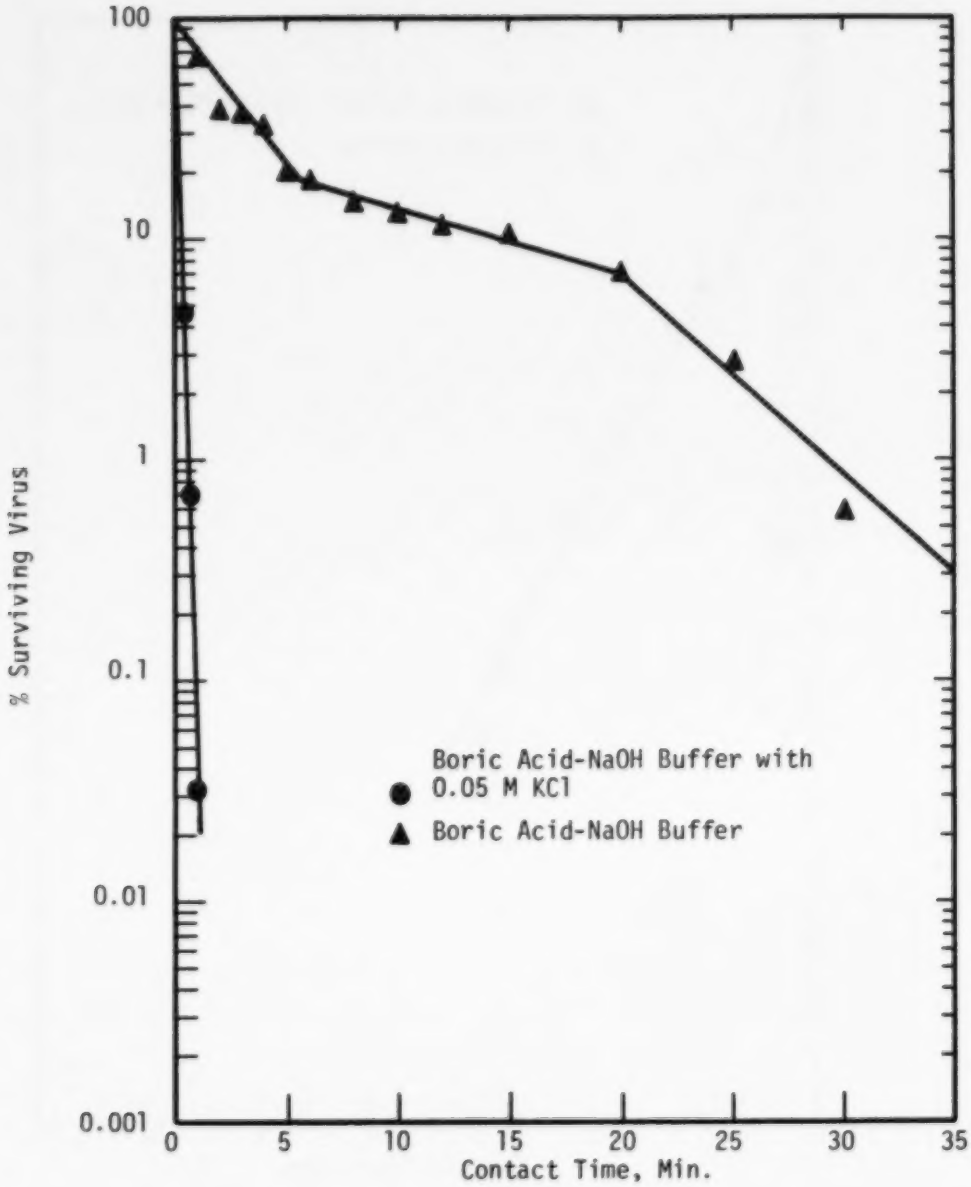


Figure 11. Inactivation kinetics of polio 1 by 0.46-0.49 mg/ℓ free available chlorine in 0.05 M boric acid-NaOH buffer, with and without 0.05 M KCl at pH 10.0 and 5.0°C.

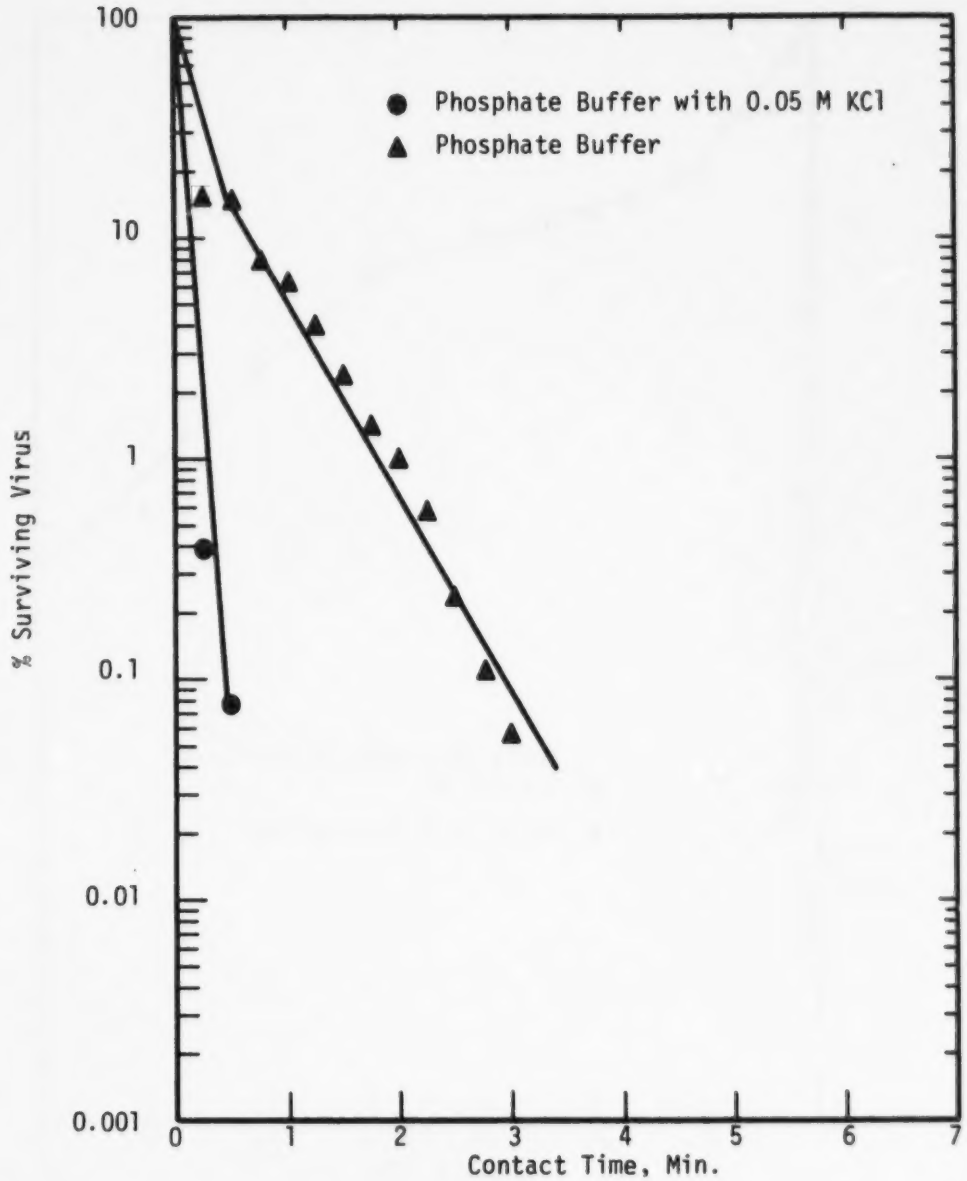


Figure 12. Inactivation kinetics of polio 1 by 0.48-0.49 mg/ℓ free available chlorine in 0.05 M phosphate buffer, with and without 0.05 M KCl at pH 6.0 and 5.0°C.

inactivation of polio 1 by free available chlorine.

Polio 1 was also used to study the effect of calcium and magnesium on chlorine inactivation. These experiments were performed at 5°C, and with 0.05 mg/ℓ free available chlorine in pH 6.0 phosphate buffer using different concentrations of magnesium and calcium. Floyd and Sharp[17] showed by electron microscopy that polio 1, suspended in 0.05 M phosphate buffer at pH 6.0, was markedly aggregated. However, this aggregation was found to be sensitive to the ionic strength of the solution, and could be prevented by appropriate concentrations of $MgCl_2$, i.e., at least 0.01 M $MgCl_2$ was required to inhibit aggregation at pH 6.0. Figure 13 shows the inactivation of polio 1 by 0.5 mg/ℓ of free available chlorine at pH 6.0 in 0.05 M phosphate buffer, with and without 0.01 M $MgCl_2$. Chlorine inactivation with only phosphate buffer shows a biphasic curve, while inactivation performed in phosphate buffer plus 0.01 M $MgCl_2$ demonstrates first-order kinetics. It appears likely that $MgCl_2$ does inhibit the formation of aggregates in phosphate buffer at pH 6.0 and this, in turn, alters the inactivation kinetics.

Using an electron microscope, Floyd and Sharp[17] observed that polio 1 (7×10^{10} particles/mℓ) in a phosphate buffer solution with 0.001 M $CaCl_2$ did not produce aggregation, but in 0.01 M $CaCl_2$ aggregation occurred. Using phosphate buffer at pH 6.0, the highest concentration of $CaCl_2$ that could be studied without the formation of insoluble calcium phosphate was 0.002 M. Figure 14 compares the inactivation of polio 1 in pH 6.0 phosphate buffer, with and without 0.002 M $CaCl_2$. There appears to be no significant difference between the two systems; both appear biphasic in nature.

It is possible that the presence of calcium ions causes increased aggregation. This is evidenced by the rate change in inactivation when polio 1 is suspended in phosphate buffer with $CaCl_2$ (Figure 14). In the system with $CaCl_2$, the rate change occurs at 30 percent survival; while in the absence of $CaCl_2$, this does not happen until survival is 18 percent. These results, although limited, suggest that magnesium might inhibit aggregation of polio 1, while calcium might tend to maintain or increase the aggregation of the virus at pH 6.0. However, to substantiate this observation, further investigation is required.

EFFECT OF AGGREGATION ON CHLORINE DISINFECTION

Electron microscope studies to determine viral aggregation were initiated. Unfortunately, the studies could not be completed because of the inaccessibility of the electron microscope. Before this situation developed, however, a few electron micrographs were taken of the polio 1 virus particles on a grid prepared by the phosphotungstate negative staining technique. The majority of the virus particles appeared to be singles as shown by electron micrograph A in Figure 15. Electron micrograph B, Figure 15, shows two virus particles that, at first, appear to be aggregated, but upon closer examination appear not to be touching each other. After looking at this grid and others, it was concluded that only a small percentage (approximately 1 percent) of the viruses in this particular preparation were aggregated.

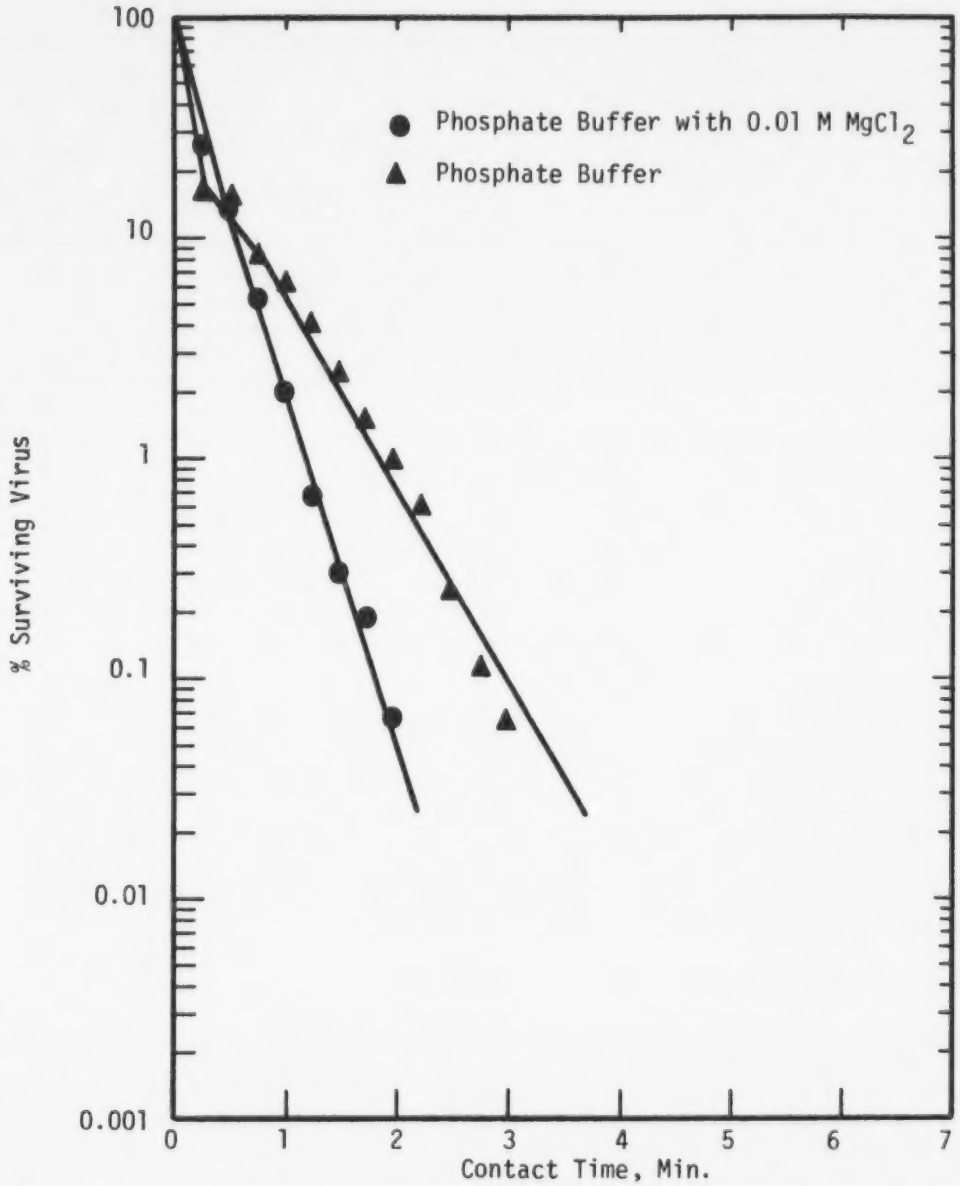


Figure 13. Inactivation kinetics of polio 1 to 0.50-0.55 mg/ℓ free available chlorine in 0.05 M phosphate buffer, with and without 0.01 M $MgCl_2$ at pH 6.0 and 5.0°C.

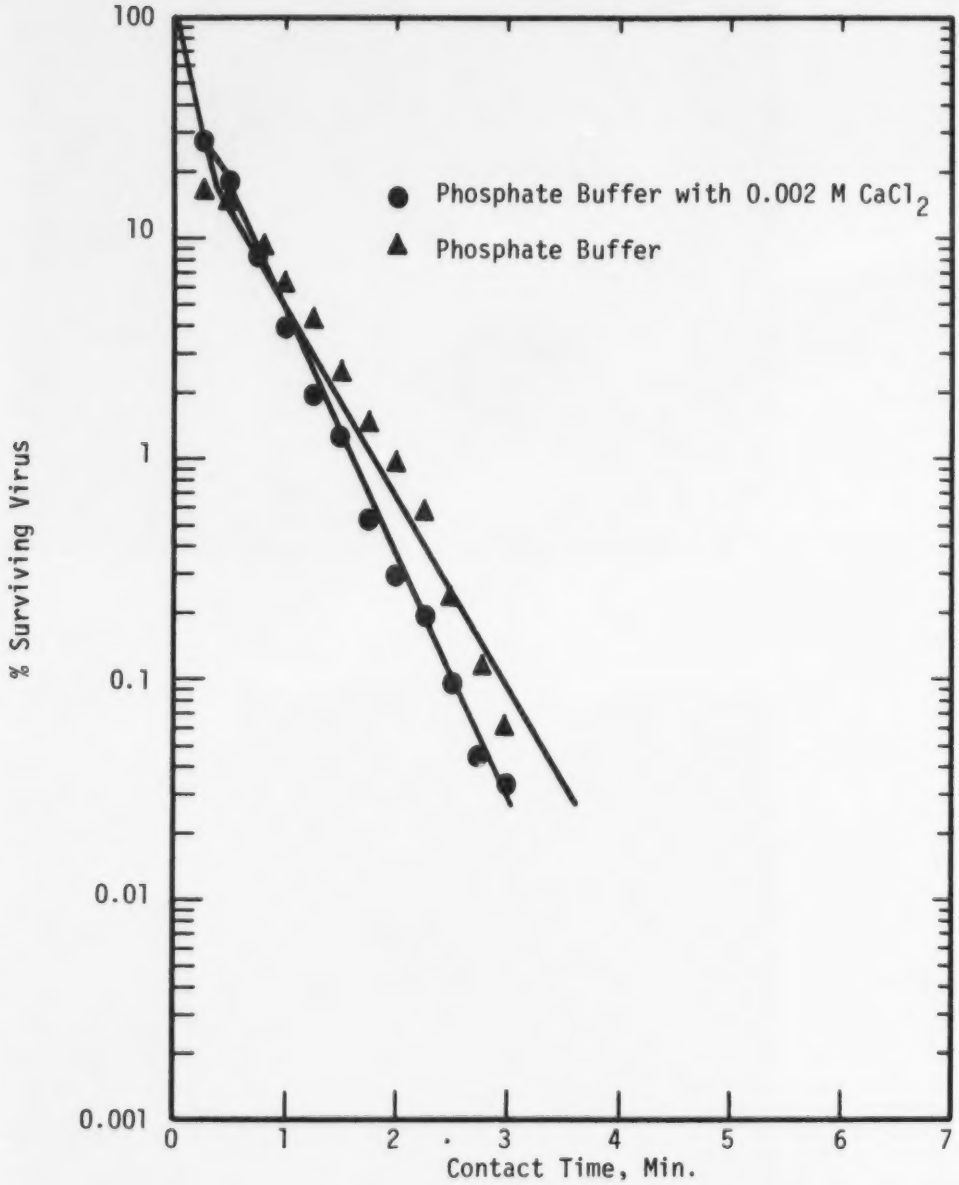
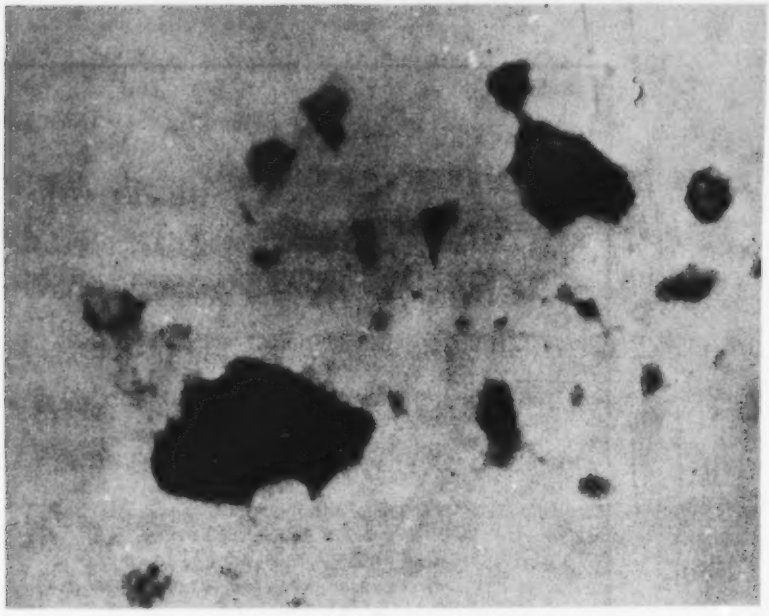


Figure 14. Inactivation kinetics of polio 1 to 0.50-0.55 mg/ℓ free available chlorine in 0.05 M phosphate buffer, with and without 0.002 M $CaCl_2$ at pH 6.0 and 5.0°C.

Micrograph A



Micrograph B

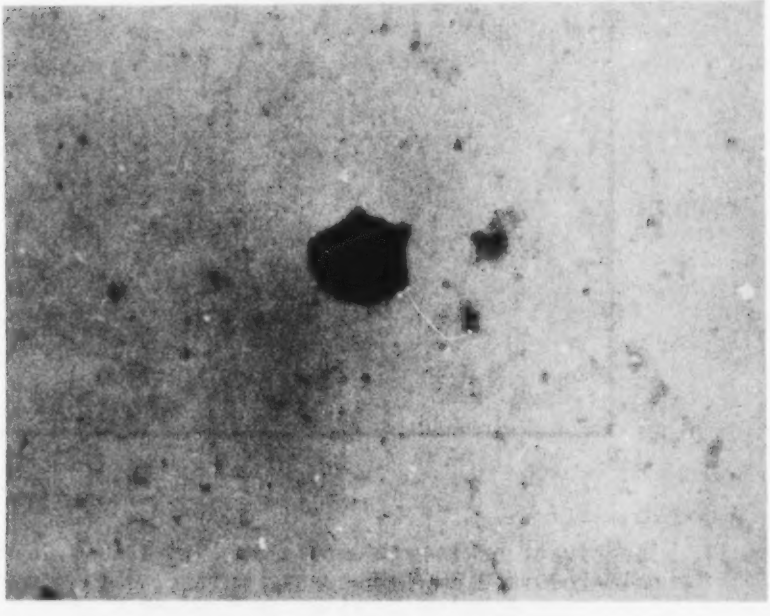


Figure 15. Electron micrographs of polio 1 by the phosphotungstate negative staining technique

Sucrose density gradients were also used in an attempt to determine the aggregation state of the polio 1 stock preparation employed in the inactivation experiments. The experiment consisted of three separate 5-30 percent sucrose gradients. The first gradient was layered with a ribosome marker (70 S), the second gradient was layered with a sample of polio 1 (150 S) used in the chlorine inactivation experiments, and the third gradient contained the same polio 1 preparation but treated with 0.001 M EDTA.

The EDTA was added to the sample of polio 1 to break up aggregates by chelating divalent cations. After centrifugation at 40,000 rpm for 30 min, the gradients were collected into 0.33 mℓ fractions and each fraction was titered by plaque assay. The results are shown in Figure 16. In plotting the density of virus in each fraction against the fraction number (40 fractions), aggregated virus should appear as either a separate peak from the fraction having single virus particles, or as a shoulder on the heavy side of this single virus peak. In Figure 16, the single virus peak for untreated polio 1 (150 S) should be approximately fraction number 26, based upon that for the known ribosome preparation (70 S).

The untreated polio 1 gradient (Figure 16) shows a peak at fraction 22, presumably consisting of single particles; this is further down the gradient than expected. Likewise, polio 1 treated with 0.001 M EDTA shows a peak at fraction 14. It would appear that treatment with 0.001 M EDTA did not break up the aggregates as anticipated. Further studies would be necessary to ascertain the significance of these preliminary results. Assuming that the major peak of the untreated polio 1 is composed of single virus particles, then possibly the shoulder on the side close to the bottom of the gradient may be due to aggregates. The shoulder accounts for about 8 percent of the total area under the peak in the gradient.

Inactivation of the polio 1 virus sample used in the above gradient studies with 0.5 mg/ℓ free available chlorine at 5°C is shown in Figure 17. The results indicate the possibility of multi-hit kinetics of inactivation; this is consistent with the presence of viral aggregates, i.e., about 8 percent of the surviving virus.

The chlorine sensitivity of various fractions from a sucrose gradient fractionation of polio 1 and echo 1 was determined. Each virus was extracted with Freon 113 and centrifugation through a 5-20 percent sucrose gradient was performed simultaneously on each virus. The gradients were fractionated into 3 mℓ fractions and each was titered by plaque assay. A gradient profile with a virus peak was obtained from each gradient as shown in Figures 18 and 19.

Representative fractions were selected from different areas along the virus peak: the left (bottom) shoulder, the peak, and the right (top) shoulder. In the case of echo 1 (Figure 19), a distinct shoulder was evident on the side of the peak closer to the bottom of the gradient. Believing this to represent aggregates, a fraction (#5) in this region was also selected to be tested for chlorine sensitivity. Chlorine inactivation experiments were performed on each of the three fractions for polio 1 and on the four fractions for echo 1.

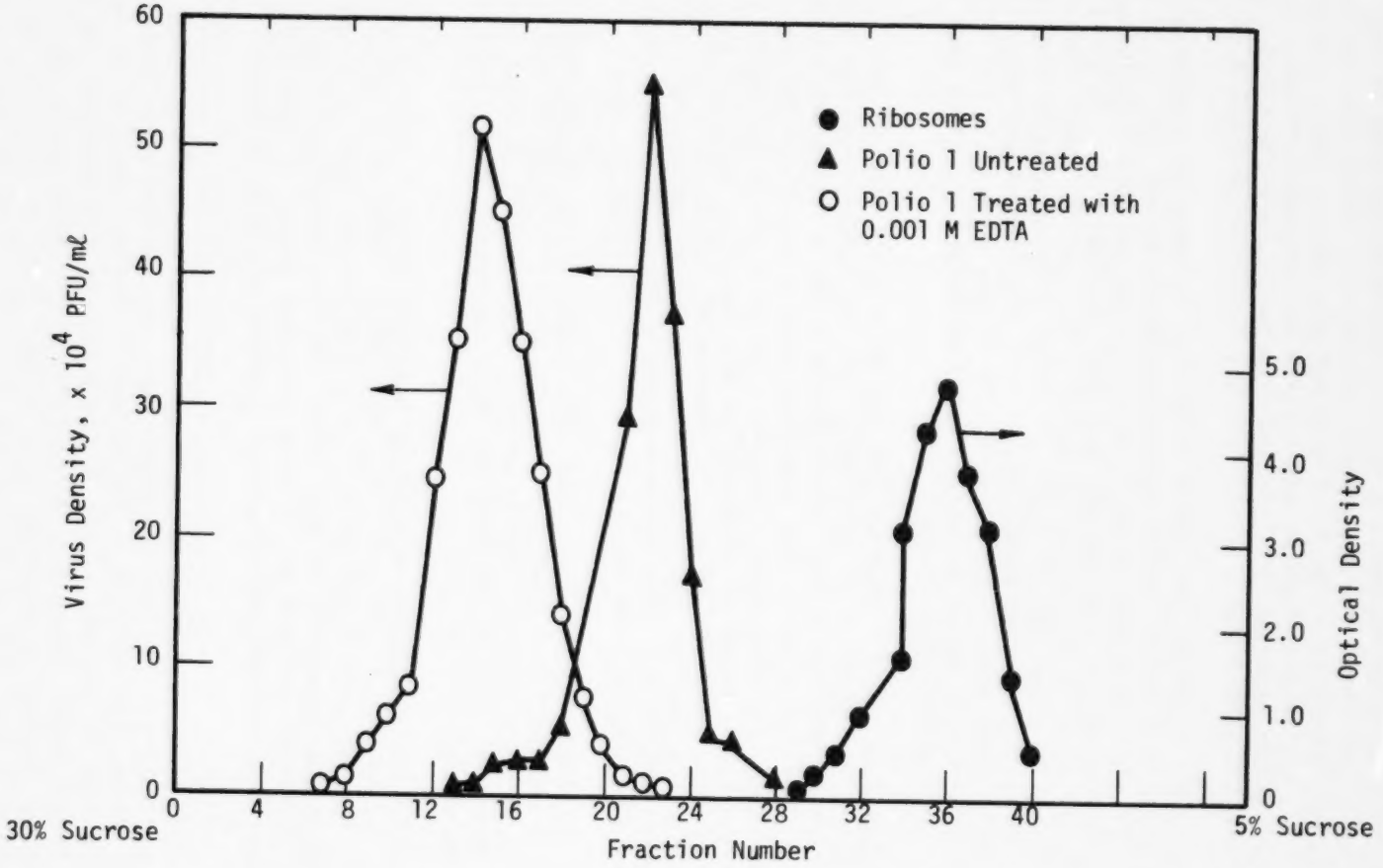


Figure 16. Fraction number vs. density of polio 1 or ribosomes in a 5-30% sucrose gradient

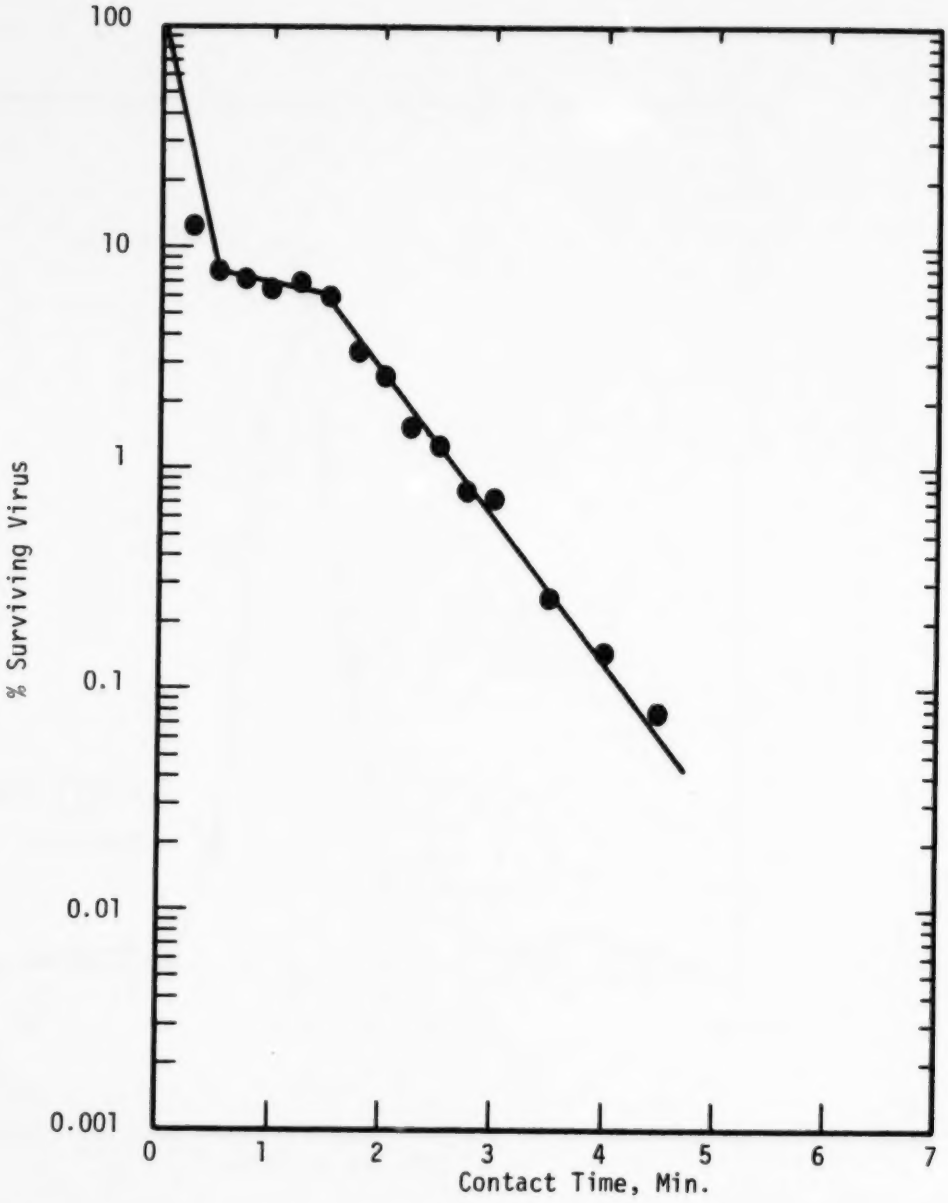


Figure 17. Inactivation kinetics of polio 1, used in the gradient studies, by 0.51 mg/ℓ free available chlorine at pH 6.0 and 5.0°C.

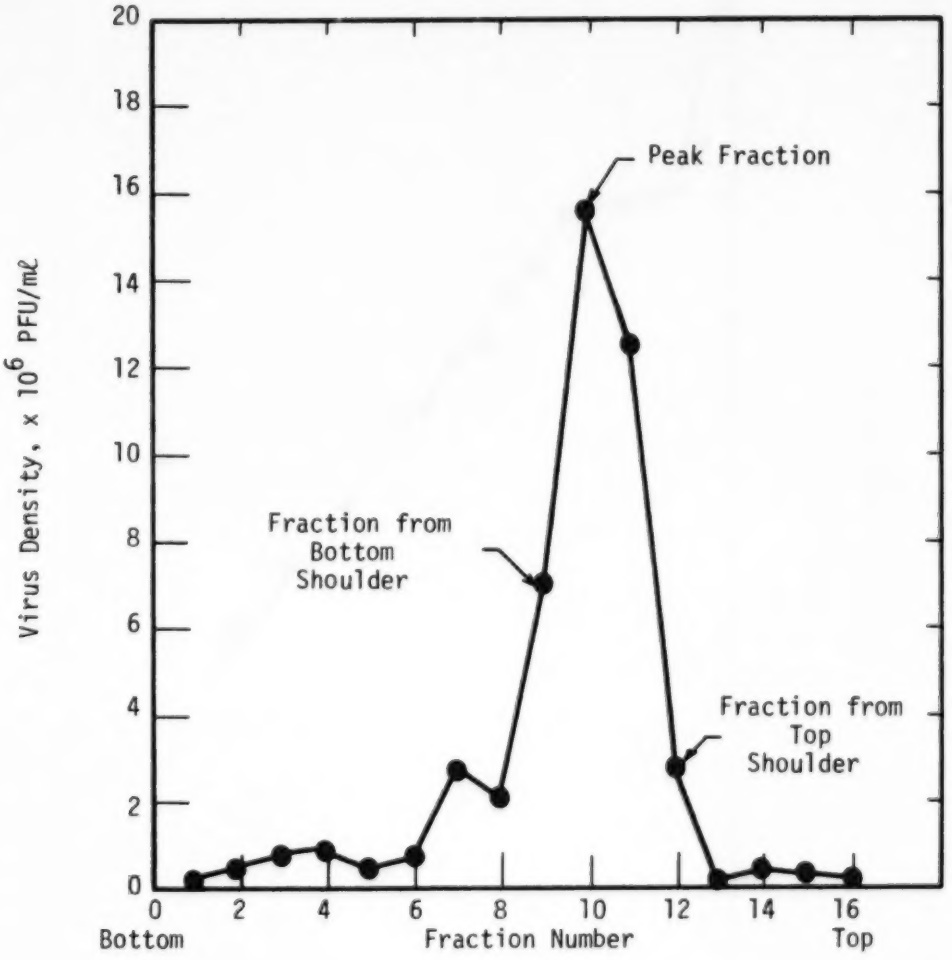


Figure 18. Gradient profile of polio 1, 5-20% sucrose gradient spun for 2 hours at 22,000 rpm at 15°C.

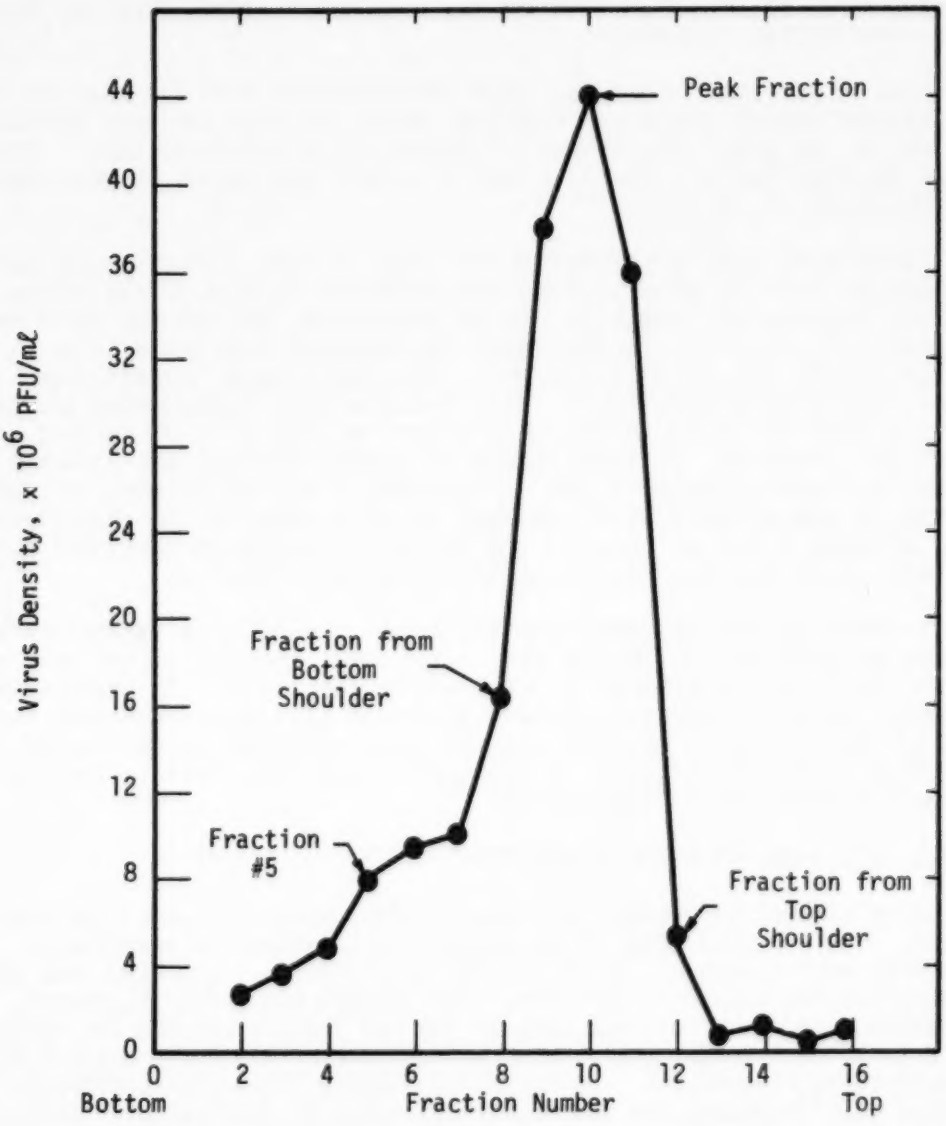


Figure 19. Gradient profile of echo 1, 5-20% sucrose gradient spun for 2 hours at 22,000 rpm at 15°C.

The results obtained with polio 1 (Figure 20) conformed to the anticipated degree of viral aggregation in each fraction, i.e., the fraction on the left or bottom shoulder, most likely to contain aggregated virus, showed the slowest inactivation (2.8 min for 4 logs inactivation), the peak fraction was more rapid (2.34 min), while the top or right fraction gave the most rapid inactivation (2.1 min).

Also of interest is the fact that the fractions from the peak and the top shoulder showed single-hit kinetics, while the fraction from the bottom shoulder of the virus peak showed a biphasic inactivation pattern. This latter fraction had possibly less than 1 percent aggregates as determined from the break in the curve.

The results with the different fractions of echo 1 showed much the same pattern as with polio 1, i.e., the fractions closest to the bottom required the greatest length of time to inactivate, and the top fraction the least (Figure 21). Fraction number 5, selected from the distinct shoulder on the left of the virus peak, gave the slowest inactivation, i.e., 4 logs inactivation in 2.90 min. This fraction also demonstrated biphasic kinetics, with the break in the curve occurring when about 20 percent of the virus still remained. A larger amount of aggregated virus was probably present in fraction number 5 than in the other fractions of echo 1. The fraction on the bottom side of the peak showed a break in the inactivation curve at about 1 percent survival and the peak fraction at about 0.4 percent survival, while the top fraction showed single-hit kinetics.

In these sucrose gradient studies, there is a definite relationship between the position of the fraction on the virus peak (or on the gradient), and its inactivation response to free available chlorine. By pooling the different fractions (fraction numbers 9 through 11), more consistent inactivation results can be obtained but the virus particles in the pooled sample are probably in various states of aggregation and, as a result, tend to demonstrate multiphasic inactivation.

GENETIC SELECTION FOR CHLORINE-RESISTANT VIRUS

In an attempt to obtain chlorine-resistant virus variants, ten plaques of polio 1 were picked after three successive inactivation experiments. The virus from each plaque was grown and stocks prepared. Each stock was then examined for resistance to chlorine. The contact times for 99 percent inactivation of the virus from each of the ten plaques were in the range of 0.2 to 1.4 min longer than the parent culture. This difference is not believed to be significant. Following completion of these experiments, Bates *et al.*[18] reported the progressive increase in resistance of polio 1 (LSc) to free available chlorine as a result of repeated exposure of the virus to a sublethal concentration of chlorine.

Polio 1 was also exposed to ultraviolet (UV) light in hopes of causing a mutation which, in turn, would produce a chlorine-resistant variant. Using a virus stock suspended in phosphate buffer, aliquots were withdrawn following various times of exposure to UV and the UV exposed samples were plaque assayed. A sample showing about two logs inactivation, i.e., after

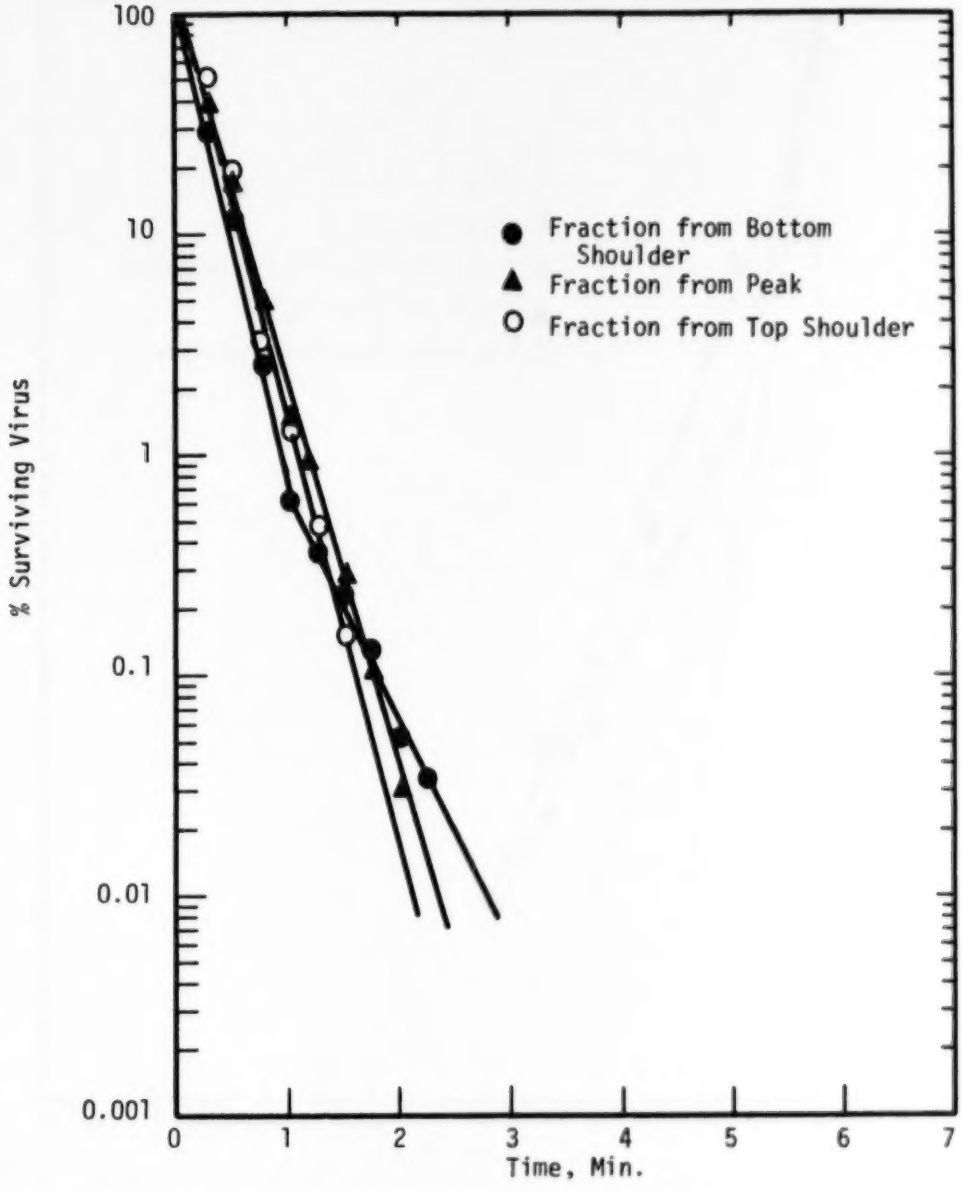


Figure 20. Inactivation of three fractions from the polio 1 gradient profile by 0.49-0.51 mg/ℓ free available chlorine at pH 6.0 and 5.0°C.

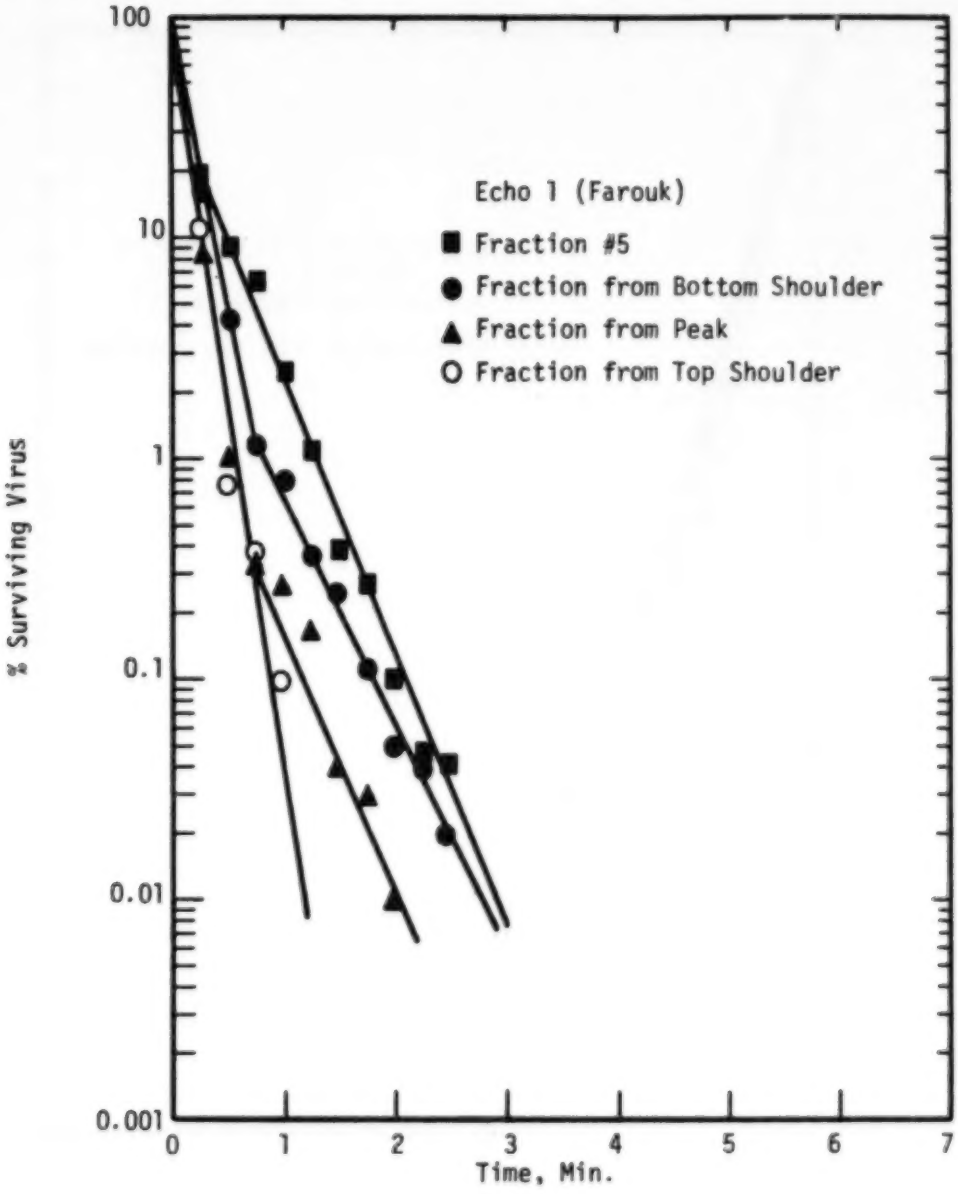


Figure 21. Inactivation of four fractions from the echo 1 gradient profile by 0.50-0.51 mg/ℓ free available chlorine at pH 6.0 and 5.0°C.

1.5 to 2.0 min exposure to UV light, was grown up in BGM cells and subsequently purified by Freon 113 extraction and sucrose gradient. A chlorine inactivation experiment was performed on the purified virus stock. A sample showing two to three logs inactivation (2.75 to 3.50 min) was selected and again grown on BGM cells, purified, resuspended in phosphate buffer, and re-exposed to UV light. Two exposures to UV light and chlorine were performed. The contact times for 99 percent inactivation were in the range of 1.6 min, which is somewhat more rapid than the parent culture. This difference, however, is not believed to be significant.

REFERENCES

1. Brazis, R. A., *et al.* Special Report to Department of the Navy, Bureau of Yards and Docks: Sporicidal Action of Free Chlorine. USPHS Report, R. A. Taft Sanitary Engineering Center, Cincinnati, OH, 1957.

2. Trask, J. D., *et al.* Chlorination of Human, Monkey Adapted and Mouse Strains of Poliomyelitis Virus. Amer. Jour. Hyg., 41:30, 1945.

3. Ridenour, G. M., and Inglos, R. S. Inactivation of Poliomyelitis Virus by Free Chlorine. Amer. Jour. Pub. Health, 36:369, 1946.

4. Clarke, N. A., and Kabler, R. W. The Inactivation of Purified Coxsackie Virus in Water by Chlorine. Amer. Jour. Hyg., 59:119, 1954.

5. Clarke, N. A., *et al.* The Inactivation of Purified Type 3 Adenovirus in Water by Chlorine. Amer. Jour. Hyg., 64:314, 1956.

6. Weidenkopf, S. Inactivation of Type 1 Poliomyelitis Virus with Chlorine. Virology, 5:56, 1958.

7. Clarke, N. A., *et al.* Human Enteric Viruses in Water: Source, Survival and Removability. In: Proceedings of the First International Conference on Water Pollution Research, Pergamon Press, London, 1962. p. 523.

8. Kelly, S. M., and Sanderson, W. W. The Effect of Chlorine in Water on Enteric Viruses. Amer. Jour. Pub. Health, 48:1323, 1958.

9. Kelly, S. M., and Sanderson, W. W. The Effect of Chlorine in Water on Enteric Viruses, II. The Effect of Combined Chlorine on Poliomyelitis and Coxsackie Virus. Amer. Jour. Pub. Health, 50:1, 1960.

10. Scarpino, P. V., *et al.* A Comparative Study of the Inactivation of Viruses in Water by Chlorine. Water Research, 6:959, 1972.

11. Liu, O. C., *et al.* Relative Resistance of Twenty Human Enteric Viruses to Free Chlorine in Potomac Water. In: Proceedings of the 13th Water Quality Conference, Virus and Water Quality: Occurrence and Control, University of Illinois, Urbana, IL, Bull. 69(1):171, 1971.

12. McDermott, J. H. Virus Problems and Their Relation to Water Supply. Virginia Section, Amer. Water Works Assoc., October 25, 1973.

13. American Public Health Association. Standard Methods for the Examination of Water and Wastewater. 13th ed., 1971. p. 387.

14. Culp, R. L. Breakpoint Chlorination for Virus Inactivation. Jour. Amer. Water Works Assoc., 12:699, 1974.

15. Sharp, D. G. Virus Particle Aggregation and Halogen Disinfection of Water Supplies. EPA-600/2-76-287, U.S. Environmental Protection Agency, 1976.

16. Floyd, R., Johnson, J. D., and Sharp, D. G. Inactivation by Bromine of Single Poliovirus Particles in Water. Appl. and Environ. Micro., 31:298, 1976.

17. Floyd, R., and Sharp, D. G. Aggregation of Poliovirus and Reovirus by Dilution in Water. Appl. and Environ. Micro., 33(1):159, 1977.

18. Bates, R. C., *et al.* Development of Poliovirus Having Increased Resistance to Chlorine Inactivation. Appl. and Environ. Micro., 34:849, 1977.